

Has the real Bush stood up?

President George Bush has won just praise for his performance in Europe last week, but the difficult decisions remain unmade, not just in Washington, but in London and Paris.

PRESIDENT George Bush's several statements on European security last week have been welcomed warmly not merely by allies of the United States, but at least in part by the Soviet Union, which is how it should be. That Bush may have been driven to some of the things he had to say by the need to compromise with West Germany on the North Atlantic Treaty Organisation (NATO)'s position on short-range nuclear missiles in Central Europe does not detract from the importance of what he had to say. Nor does it matter that Bush appears to have been jockeyed into the position he took up last week by the crying need that the United States should somehow respond to the noises Mr Mikhail Gorbachev has been making in the past four years about arms control in Central Europe (and, for that matter, elsewhere). In this business, what matters is not rhetoric but binding agreements. How they are reached is strictly irrelevant.

The new position of the United States has four essential elements. First, the United States has agreed to match the Soviet Union's declared intention of reducing its conventional forces in Central Europe with smaller reductions of its own; the details have not been worked out (and the need to do so has apparently taken NATO officials by surprise), but this paves the way for rapid progress in the negotiations now under way in Vienna. Second, the United States accepts that aircraft should be counted in the conventional arms equation, which is as it should be (British and French views notwithstanding). Third, the United States now accepts that there should be negotiations with the Soviet Union on short-range nuclear weapons in Central Europe, but only when there is an agreement on conventional forces for which Bush demands a timetable of "six months to a year"; this is the West German compromise, about which the British and French governments are even less happy. Then, finally, there is an element of non-empty rhetoric: there can be no "common European home" (Gorbachev's phrase) until the Berlin wall has been dismantled figuratively as well as physically.

The implications must be far-reaching. The now-better prospect that there will be a deal on conventional arms in Europe means that both the major powers will win considerable economic benefits. The Soviet Union will be the chief beneficiary, but the impending arrangement should make (or at least make possible) significant inroads into the US budget deficit. Other members of the two alliances will benefit similarly. But, even more important, there is a

good chance of a continuing process of arms control in Europe. If the process is not as quick as Gorbachev has been looking for, it is a prospect that would have seemed unthinkable even a few years ago. Nobody in his senses can complain.

So what are the difficulties? The most obvious are those already made plain by the British and French governments, both of which are opposed to the counting of aircraft in the conventional arms equations and which are deeply uneasy now that they know that it is only a matter of time before their nuclear weapons must be put up for negotiation. But this is unavoidable. The notion that the two governments might be free to deploy short-range nuclear weapons (on aircraft) as they choose while the major powers are negotiating limits on what they can deploy is, of course, preposterous. Better that they should face up now to the reality they may not be able to avoid in 1990. But Britain and France will have to accept, perhaps when the START talks on strategic weapons begin later this month, that their submarine forces will also have to be counted in the equations on strategic missiles. That is what happened implicitly during the negotiation of the never-ratified SALT II treaty a decade ago. This time, the counting will be explicit. The British and French governments had better reconcile themselves to that. Deciding what to aim for will not be easy. The more submarine missiles they insist on keeping, the less will be the influence of the United States in Europe.

The other obvious difficulties are the uncertainties created by the new circumstances. Bush's West German compromise will probably suffice to keep West Germany a solid member of NATO, at least if the timetable for negotiations on conventional arms can be kept. But it will be a different kind of NATO — one in which the US physical presence is significantly though not substantially diminished and in which smaller powers will have a bigger say. Ten years from now, especially if West European dreams of economic growth through cooperation come true, its military dimensions could be much less obtrusive than at present. The uncertainties in the East are greater. Bush's challenge about the Berlin Wall will have struck many chords in Poland, Hungary and the Baltic States, but Gorbachev's demeanour in the past few months suggests that he, at least, is sympathetic. But will he be able to carry his colleagues with him? If so, the result could be that Europe is radically transformed. □



Unstable money

The turmoil in the foreign exchange markets calls for more durable remedies than now exist.

THE continuing instability of the international currency markets seems to have taken even the world's financial institutions by surprise. Over the past three years, there have been regular meetings of the chief central banks at which the participants have smugly reaffirmed their determination to keep exchange rates stable and their confidence that they could do so. But during the past nine months, the US dollar has been riding a kind of switch-back, first falling as people with cash to invest in other people's currencies took fright at the prospect that the US federal deficit would persist, and then rising, like water running uphill, as the same people found encouragement in signs that the remarkably flexible economy of the United States might keep on growing (which is the only remedy for the federal deficit that the Bush administration offers). But now, in the past few days, the dollar has started falling again as the first signs of recession make their appearance. What is going wrong?

The dismal science of economics may on this occasion surely be looked to for an explanation. The world's central banks have been seeking to play a confidence trick on the international community of those who invest in other people's currencies, mostly commercial banks and other financial institutions. By saying they will and can keep exchange rates constant, the central banks have been trying to persuade people that there are no prizes to be won by betting that particular currencies would rise or fall. If people had believed them, their assertion would have come true. In a vacuum, so to speak, the trick might have worked. In the real world, it has not.

Nobody should be surprised. Central banks have only one durable means of affecting the value of their own currency — the rate of interest they require their own commercial banks to pay for money borrowed. Higher interest rates will sustain the value of a currency, lower interest rates work the other way. But the same interest rates affect the real economy. In the long run, they depress economic activity. In poor Britain, the Bank of England's rate has already reached 15 per cent, and may yet go higher, for the British have to persuade other people to hold sterling by compensating them not merely for tying up their money but for the rate at which the value of their assets will be eroded by inflation, now eight per cent (in Britain). In the United States, where inflation runs at a lower rate, lower interest rates still threaten to bring on recession (which is why the US dollar is now falling). The most likely consequence of the banks' confidence trick will be a general recession next year.

Might there have been a better way? The pressure on the foreign exchanges is a measure of the imbalance in world trade. The yen the Japanese earn by their ingenuity do not simply stay in the pockets of their manufacturers

but are mostly converted into other currencies by the Japanese commercial banks. After many years of trade imbalance, these sums of money are much greater than can be converted into long-term assets by their owners, which explains why short-term interest rates are now higher than long-term rates — and the foreign exchange markets are uncomfortably volatile as a result. So the central banks should be working harder than they are at increasing long-term interest rates even if that implies that debtor nations as the United States and Britain would be more painfully punished for profligacy. □

China in trouble

The past month's turmoil in China may eventually benefit Chinese science, but the outlook is not cheerful.

CHINA is once more in upheaval. The economic reforms of the past decade, now themselves in hazard, have chiefly affected the peasant farmers able to sell what they produce on a relatively free market and the small urban entrepreneurs who have been allowed to recreate some part of traditional Chinese small business. But university teachers and the army of workers in China's research institutes have been almost untouched by the unwinding of the Cultural Revolution. It is still the norm that people are paid miserable salaries and are expected not to complain too bitterly on the grounds that their housing is almost literally free and, by the same token, is a means by which they are kept captive. There has been some progress in persuading technical people that they might be happier and more productive working more closely with industrial enterprises, but most of the over-large research institutes are still too encumbered with dead wood and ideology. It is not surprising that the promise that China's science would contribute both to the enrichment of China and to general understanding has not materialized.

What will happen now that the academic community's students have taken the lead in a peaceful protest against lack of liberty, and when some of its members are being punished for their effrontery? It is natural that university students should have been in the van of the recent protests; they after all, have the most to lose from a continuation of the sloth that has overtaken the once-reforming leadership in Beijing. It seems unlikely that China's researchers will have much to gain from what happens in the months ahead. But the privations of daily life in China breed fortitude, while the still-fresh legend of the Cultural Revolution is not just a tale of repression, but also a rich anthology of tales of individual heroism and ingenious defiance. That should help keep some people's spirits up. It is also relevant that the weakness that has sapped the resolution and imagination of the Beijing regime is intrinsically self-limiting: even in China, octogenarians cannot hope to live forever. Meanwhile, it is essential that the links with Chinese science forged in the past decade should be kept intact, even strengthened. □

Raids cause French workers to take stock

- Break-ins may set back research by five years
- Animal welfare committee to be created

Paris

Two weeks after anti-vivisectionists raided research laboratories in Lyons, stealing more than 100 animals, the potential implications for French biomedical research are beginning to sink in. The animal rights lobby has posed little threat to researchers in France and there have been only two raids on animal houses in the past ten years. But now the national medical and health research institutes (INSERM) to which the Lyons laboratories belong realize that they are ill-prepared to defend themselves either in terms of security or in the arena of public debate.

Shortly after the break-in at INSERM units 94 and 37 during the night of 20 May (see *Nature* 339, 326; 1 June 1989), a hitherto unknown group called Arche de Noé (Noah's Ark) telephoned the news agency AFP to claim responsibility. The following evening, national television news (Antenne 2) broadcast a macabre video-recording of the raid, filmed by Arche de Noé, together with statements by members of the group. Last weekend, a popular magazine, VSD, published colour photographs of the break-in and a chronology provided by one of the group. INSERM has called for charges to be brought against the perpetrators for forced entry, theft and damage to government property. But French police, unused to dealing with this kind of crime, have so far been frustrated in their attempts to seize the videotape and to arrest the self-declared culprits.

INSERM Unit 94's work was recently published in *Nature* (337, 265; 19 January 1989) and subsequently criticized by an advisor to the British Home Office (see below). But, despite the coincidence, the INSERM laboratory may have been singled out for attack because of its links with Colin Blakemore, Waynflete Professor of Physiology at the University of Oxford. Blakemore, who has been the prime target for anti-vivisectionist attacks (including a death threat) for the past few

years in Britain, has collaborated with the Lyons laboratory for the past 14 years (see page 414).

"There are well-established links between animal rights groups around the world", says Blakemore, who explains that a newsletter called *The Liberator* regularly publishes lists of names and addresses of contacts as well as tactics. "I think they just spotted a growing connection between Oxford, which is the focus of hatred in England, and the Lyons laboratory, which happens to be one of the internationally best-known labs in neuroscience." Several of the 38 monkeys stolen from Lyons had been used in experiments carried out by Blakemore at Unit 94.

The raid on the adjacent Unit 37, where organ-transplant research is carried out, often on dogs, coincides with a rise in public support for animal rights. France boasts an estimated 10 million pet dogs for its 55 million population and has a popular animal rights champion in Brigitte Bardot, who only a few weeks ago presented her views in a television broadcast. A bill to improve the law relating to domestic pets is currently passing through parliament.

The Lyons raid raises questions about differences within Europe in laws governing animal experimentation. Mr Clive Hollands of the Committee for the Reform of Animal Experimentation has claimed that the experiments, which involved the blinding of two macaque monkeys before birth, caused "an unacceptable level of suffering" and "clearly would not have been authorized under British law" (*Nature* 339, 248; 25 May 1989), but this is a matter of opinion, as every British application is judged on the balance of its biomedical benefits against the suffering that would be experienced by the animals. One researcher at INSERM Unit 94 agrees that the French law is less severe than the new British law. But, he says, similar experiments have been carried out in the United States and have been published in the journal *Science*.

In France, animal experimentation is regulated under a 1987 Act which follows guidelines developed by the European Parliament. Under this law, once a researcher has been authorized by the Minister of Agriculture to carry out animal experiments, his licence is valid for ten years. Similarly, approval of laboratories and animal houses is granted for five years.

The law places responsibility on the licensed researcher, says Professor Pierre

Tambourin, who has been delegated by the director general of INSERM to co-ordinate animal experimentation policy matters. Tambourin admits that the licence is open to abuse: "there is nothing in the law to stop a madman doing anything he wanted to an animal", but points out that this is true everywhere. Each INSERM unit undergoes a detailed in-house review every four years, he says, and peer review of papers submitted for publication also serves to control ethical issues.

According to Blakemore, it is common practice for reviewers of leading neuroscience journals (he cites *Brain Research* and the *Journal of Neuroscience* as examples) to be asked to comment on the animal welfare aspect of a study. *Nature*, he says, does not do this.

Faced with a demand to justify animal experimentation, INSERM is discovering it has no official documentation and no cogent arguments. "The benefits were always taken for granted", says Tambourin. The Lyons animal houses are on open view to those visiting patients at the adjacent hospital. Blakemore says it was "wonderfully refreshing" to work in the Lyons laboratories, adding that in 14 years there have been no complaints from the public.

But there are no immediate plans at INSERM to introduce the level of security now necessary in Britain and the United States. "Of course we will replace locks and install alarms", says Tambourin. "It's stupid. We have nothing to hide. It is a negation of our own ethics. Research must be open permanently to the public." A national committee for animal welfare is being set up with representation from the Society for the Protection of Animals as well as researchers, in addition to the existing national animal experimentation commission which was established under the 1987 law.

The loss at the Lyons laboratories is estimated at more than FF1 million (\$150,000). But research there has been set back by five years, according to one researcher — the time it takes to breed animals and train them for experiments. All the laboratory notes of three researchers were also stolen during the raid. He also believes that many of the stolen monkeys will not have survived. The colony included well-established social groups. If two dominant males were accidentally put together, he says, they would kill each other. In the meantime, INSERM is determined to ensure that the culprits are prosecuted, while researchers in Lyons think they have evidence that the raid was planned as long ago as last Christmas.

Peter Coles

■ In Australia a new code of practice on animal experiments is to be implemented in response to pressure groups. See page 412. □

Announcement

Joseph Palca, Washington News Editor, leaves *Nature* this week to join the US journal *Science*. He will be joined by Marcia Barinaga, *Nature's* West Coast correspondent, who was recruited by *Science* last month. At *Science* both are expected to perform similar roles to the ones for which they were greatly appreciated at *Nature*. □

New technique on trial

Washington

PRESSURE to set new national guidelines for the forensic use of genetic fingerprinting techniques and to re-examine old trials in which DNA analyses provided key evidence is growing in the wake of controversial testimony given at a double murder trial in the Bronx, New York. A string of expert witnesses at the trial questioned the way laboratories carry out DNA analysis and interpret and match their data.

Last week, the two defence attorneys in the case, Barry Scheck and Peter Neufeld, said that they had formed a committee with the National Association of Criminal Defense Lawyers to "serve as a clearing house and help people reopen cases" involving Lifecodes Corporation of Valhalla, New York, the company that carried out the DNA analysis in the Bronx case. Scheck, Professor of Law at the Benjamin Cardozo School of Law in New York, said he has received calls every day from lawyers across the country who are desperate for help. Most calls received so far concern pending cases.

At the Bronx trial Jose Castro, a 38-year-old Hispanic, is accused of murdering two neighbours, a woman and her infant daughter. According to a report prepared by Lifecodes for the prosecution, a spot of blood on Castro's watch has a DNA fingerprint matching that of the murdered woman. But at a pretrial "Frye" hearing to examine the admissibility of the evidence, an impressive line-up of geneticists and molecular biologists, including Eric Lander and David Page of the Whitehead Institute, Conrad Gilliam of Columbia University and Howard Cooke of the Medical Research Council in Edinburgh, raised serious doubts about Lifecode's analysis (*Nature* 339, 89; 11 May 1989).

Those doubts culminated in an informal "scientific" meeting of expert witnesses, including molecular biologist Richard Roberts of Cold Spring Harbor laboratories, who had given evidence for the prosecution. Their consensus view was that the data in the case were not scientifically reliable and would not be accepted by a peer-reviewed scientific journal.

The pre-trial hearing is now over and a ruling from Judge Gerald Sheindlin is expected next month. The judge could make a general ruling on DNA fingerprinting, perhaps saying that it is admissible if proper standards are set, or perhaps taking a tougher line by saying that the technique is only good enough to prove that two samples are not identical. Whether or not the judge chooses to make a general ruling, he is widely expected to decide that Lifecode's data are not admissible in this particular case.

John Winkler, Lifecode's senior vice president, refuses to comment in detail on the case, saying that "we don't want to do

anything that smacks of trying the Castro case in the press". When the company is "free to talk about the entire case we'll talk about it point-by-point", he says. Lifecode's scientists are "pretty upset about some of the things that are being flung around" in criticism of the company, Winkler says. He defends their record, saying Lifecodes has taken part in "every proficiency test we can find, we wish there were more" and provides "a good service". But Winkler agrees with the defence attorneys that there should be national guidelines for forensic DNA analysis. That, he says, is something his company has always advocated.

The most likely source of new guidelines is the National Academy of Sciences. The academy has already tried once to launch a study of the technique. John Burris, Director of the Commission of Life Sciences, is now optimistic that the academy will be able to raise sufficient funds.

DNA FINGERPRINTING

Genetic tests made official by UK courts

London

DNA PROFILING was added last week to the range of techniques available for use at the request of the British civil courts in cases of disputed paternity, and this week the Home Office may announce plans for a centrally run scheme for DNA testing in immigration cases.

The technique is already widely used in paternity cases: in 1987 (the only year for which the Home Office can provide statistics) it was used as evidence in 1,000 cases at the request of the individuals involved. From now on, the courts will be able to request a DNA test; use of the technique in legal cases is likely to increase as a result, according to a Home Office spokesman.

For 18 months, the UK government has accepted DNA profiles as evidence in immigration cases. In the past 4 months, DNA profiles were accepted as evidence in 750 cases. In 86 per cent of those, the claimed relationship proved to exist; in 12 per cent it was disproved; and in 2 per cent of the cases, the results were inconclusive.

The tests are carried out for the individual at his own expense usually by Cellmark Diagnostics, the company with exclusive rights to commercialize the test developed by Alec Jeffreys of Leicester University. When the report is submitted as evidence, the Home Office requests a copy from the company to ensure that presented by the individual is valid. A government scheme would centralize these procedures, but the costs would remain with the individual.

Use of DNA profiling is being seriously scrutinized in the United States as a result of questions raised concerning the quality of DNA analyses presented as evidence in a murder case in the Bronx (see above). But in Britain the quality of the analyses

Providing guidance on a controversial scientific topic is "what the academy can do best", says Burris.

Until proper guidelines are in place Scheck thinks that there should be a moratorium on the forensic use of DNA analysis. Lifecodes' Winkler does not agree. "The technology is valid", he says, "there is no question that the results can be interpreted correctly now".

Scheck also says that DNA analysis presents particular problems for defence lawyers because of the difficulty of finding expert witnesses. Publicity in the Castro case will, he hopes, make it easier for lawyers to find "someone literate in molecular biology and population genetics". Winkler accepts that lawyers have often not known what to do when faced with the new technology of DNA analysis but disputes that there is any shortage of expert help. Southern blotting, which lies at the heart of the technique, has been around for more than a decade, he notes.

Alun Anderson

presented as evidence in court is rarely questioned. Jeffreys cites only one case in which the DNA analysis was found to be at fault; after the test was carried out a second time a prosecution was secured.

Howard Cooke, of the Medical Research Council genetics unit in Edinburgh, says the reason why problems of quality have not been raised in Britain may be that the system is much more "closed" than in the United States. Only the government's Forensic Science Service (FSS) or Cellmark carry out DNA tests for the prosecution in criminal courts, though Cellmark is now carrying out tests mainly for the defence as the FSS expands. The monopoly of the technique by Cellmark is a problem with the British system, says Cooke, although he also says that it may be as a result of commercial pressures that companies in the United States need to produce a result even when the evidence is of poor quality.

The only other private company that carries out DNA profiling in Britain is University College Diagnostics, a company set up by University College London. Only one year old, and employing less than 10 staff, the company cannot be said yet to pose a serious threat to Cellmark. But for the first time, it is about to act for the defence in a civil case in which Cellmark is acting for the prosecution. And managing director Alan Syms claims that at a basic fee of £110 per test, the company is undercutting Cellmark's price by more than £10.

There is no official licensing system for companies that want to use DNA fingerprinting commercially. The Home Office will monitor the quality of a laboratory's work and its financial security before formally approving it and placing contracts.

Christine McGourty

MEDICAL ETHICS

Japanese doctors keep quiet

Tokyo

IN A precedent-setting court case, a Nagoya judge last week ruled that Japanese doctors are not obliged to tell patients suffering from terminal cancer the true nature of their condition. "Informed consent", the notion that a patient has a right to know before giving consent for treatment, has yet to win recognition in Japan.

The case involved Kazuko Makino, a 50-year-old woman. She went to Nagoya Red Cross Hospital in January 1983 where she was told she was suffering from gallstones, although the hospital doctor suspected gall-bladder cancer. The doctor recommended surgery, but Makino, a nurse, knew that gallstones are not life-threatening and decided against the operation. By June 1983 the cancer had spread to her liver and in December she died.

Her husband and family filed a malpractice suit against the hospital for failing to inform Makino of the true nature of her illness. But in last week's district court ruling, the judge rejected the suit concluding that Makino alone was responsible for her decision to reject surgery. Judge Kuniharu Ito ruled that although it is a doctor's duty to "explain accurately and concretely about the sickness to the patient" it is up to the doctor to decide to whom, when, what and how much to explain because such disclosure can affect the recovery of the patient.

Makino's case is not exceptional. As a general rule, doctors in Japan do not tell patients if they have cancer. In a survey of several hundred doctors by the *Mainichi* newspaper in 1987, nearly 80 per cent of doctors said that the patient should not be told. Even the late emperor was not told he had pancreatic cancer. And some doctors do not inform AIDS patients of the true nature of their condition.

The *Mainichi* survey revealed that doctors believe that their patients will not be able to cope with the truth and that telling them they have cancer will only hasten their demise. But also behind the silence lies the Japanese deference for authority. Patients tend to accept the diagnosis and treatment of their doctors without question. The Makino family is exceptional in taking their doctor to court.

The need to conceal the true nature of a cancer patient's condition also helps to explain a peculiar feature of drug sales in Japan. Unlike the United States and Europe, where anti-ulcer drugs and treatments for hypertension are the biggest sellers, Japan's pharmaceutical market is dominated by Krestin, an anti-cancer agent marketed by Sankyo Pharmaceuticals. Krestin, an extract of a tree fungus, has annual sales in Japan of around \$400 million. But it is not sold

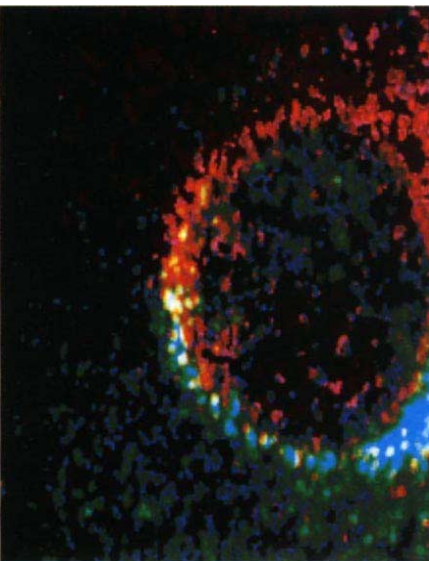
anywhere else in the world. And despite its phenomenal popularity, there are real doubts as to its efficacy.

According to Masanori Fukushima, head of the department of internal medicine at Aichi Cancer Centre, there is no conclusive evidence that Krestin prolongs the life of cancer patients. A spokesman for Kureha Chemical Industry, the company which developed Krestin, agrees that mortality of the patients was not examined.

Krestin's advantages are that it is taken in a convenient oral form and has no side-effects, causing none of the tell-tale symptoms, such as hair loss, of other anti-cancer therapies. Thus patients given Krestin can remain unaware they are being treated for cancer. And doctors who prescribe Krestin can ease their conscience by knowing they have at least prescribed something.

But times are changing. A recent report from Prudential-Bache Securities refers to Krestin as an "embarrassment to the Japanese medical community" and says that in the future Japanese patients and doctors can be expected "to cooperate in fighting cancer as they do in Western society now". Krestin, which was approved for marketing in the late 1970s, is being re-evaluated by the Ministry of Health and Welfare. **David Swinbanks**

False-colour Dawn



JAPAN'S satellite Akebono (Dawn) snapped this picture of the aurora australis from an altitude of 7,500 kilometres over the South Pole on 27 April. The false-colour ultraviolet image spans a distance of about 4,000 kilometres and the exposure time was 0.6 seconds. Akebono was launched in February by the Institute of Space and Astronautical Science (*Nature* 338, 8; 2 March 1989). □

WEST GERMAN AEROSPACE

Space plane spawns hypersonic plans

Bonn

A GRANTING committee of the Deutsche Forschungsgemeinschaft (DFG) is expected to overcome its initial misgivings and approve today two "special collaborative programmes" for research into hypersonic technologies. The DFG will invest about DM8.2 million in the three years beginning in July 1989 to recreate a West German presence in a research area that the country abandoned 15 years earlier. Two further programmes in the field are expected to be proposed by groups in Braunschweig and Stuttgart and evaluated in the summer of 1990.

The influential science council (Wissenschaftsrat) has already approved the programmes, as has a DFG committee. All that is needed is the formal approval of the special collaborative programmes granting committee, which are expected to be given on 8 June.

Both special collaborative programmes will help to train young engineers in the field of aerospace plane development. Their expertise will be needed to realize West Germany's ambitious plans to build an aerospace plane called Snger (see *Nature* 338, 105; 9 March 1989).

Three faculties in the Technical University of Aachen will investigate the feasibility and economy of various conceptions of an aerospace plane. Groups of researchers at the Technical University of Munich, at the University of the Bundeswehr (armed forces) and at the German Aerospace Research Establishment (called DLR in German) will investigate the complex physical-chemical processes involved in hypersonic engine design.

The Research Ministry plans to spend DM220 million over the next four years on developing Snger, with DLR contributing an additional DM85 million and private industry as much as DM30 million. Snger could be used for both cargo transport into space and cargo or passenger transport from point to point on Earth.

A decision is expected by 1993 on whether European efforts will focus on Snger or whether Europe will instead choose competitive planes from Britain or France. A negative decision could mean the abandonment of the project.

Opposition to the project continues from those in the physics community, especially from those who doubt that the popularity at present enjoyed by space research will yield significant long-term results. Even the Wissenschaftsrat "regrets" that there is not more coordination of research plans than is provided by these two programmes. The Wissenschaftsrat also warns that other areas of research such as orbital systems must not be neglected despite the emphasis on hypersonics. **Steven Dickman**

Uncertain start in Montreal

Montreal

THE Fifth International Conference on AIDS got off to an uncertain start this week after AIDS activists stormed the entrance to the conference centre and held up the opening ceremony for almost an hour. Conference officials stood by nervously conferring on walkie-talkies as predominantly US activists chanted slogans and read a ten-point manifesto. The activists then seized the 50 or so seats reserved for conference VIPs, shouting "We are the VIPs". Only after other space was found did some of the activists relinquish their seats, and allow the programme — which juxtaposed Canadian prime minister Brian Mulroney, Zambian president Kenneth Kaunda, and a videotape of the recently deceased founder of the Vancouver Persons With AIDS Society — to begin.

This year's conference, with a record 10,789 participants registered on the opening day, is quite different from its predecessors. The clinical, basic research and epidemiology sections are still the largest, but perhaps reflecting the failure so far to find a cure or effective vaccine, the other six sections concentrate on the effects of the AIDS pandemic on society. Sessions on the economic impact of AIDS, ethical and legal issues, and international cultural differences are interleaved with those on "AIDS and the Individual" and "AIDS, Society and Behaviour". The organizing committee, chaired

by Ivan Head of the Canadian International Development Research Centre, has a distinctly liberal bent, and includes for the first time a person with AIDS.

Head made a point in his address to acknowledge the AIDS activists in the audience: "As with all good parties, more people attend than you are prepared for", he said, adding "I am delighted you are *all* here". The opening remarks included no hints that significant developments in the understanding or treatment of AIDS were to come later in the week, but painted a grim picture of the challenge AIDS presents to society. Jonathan Mann, director of the World Health Organization AIDS programme, said the estimated half-million worldwide AIDS cases represent three epidemics: viral infection, disease and society's "extraordinarily complex and increasingly diverse" response. Mann predicted the present number of AIDS cases will double to more than 1.1 million by the end of 1991, but that over one-third can be prevented through educational campaigns, in which community-based organizations play an integral part. To loud applause, Mann issued a final challenge: "...is the world now mature enough, now wise enough to accept that the deepest meaning of solidarity requires that we consider ourselves as if we too were infected with HIV?"

President Kaunda offered a very personal note in his keynote speech, when he recounted the death of his oldest son from

AIDS more than two years ago. Kaunda emphasized that the AIDS epidemic "adds a new dimension to the destruction of the human body", particularly in Africa, where malnutrition, malaria and other infectious diseases already drastically shorten life.

In response to the question of a possible African origin for the AIDS virus, Kaunda said the seriousness of the pandemic overshadowed speculation that the virus evolved "in the tail of a monkey" or "jumped out of a test-tube of a hard-nosed researcher". Kaunda likened the AIDS epidemic to a "soft, silent nuclear bomb" that "laugh[s] at mankind and our wicked plans for self-destruction". He called for diverting the money being spent on nuclear weapons into AIDS research, saying "AIDS can do that killing for man worldwide at no cost in dollars and roubles."

Carol Ezzell

UK UNIVERSITIES

End to exam boycott

London

BRITISH university lecturers have narrowly voted to accept the pay settlement agreed last month between the university management and the lecturers' union, bringing to an end the five-month boycott of examinations which had threatened to delay the awarding of degrees to final-year students.

On a turnout of 68 per cent of the 30,000-member union, 55 per cent voted in favour and 45 per cent against the settlement, which gives lecturers a rise of 6 per cent from 1 April and a lump sum of between £150 and £285 depending on what each university can afford.

The lecturers' action began in January with a refusal to cooperate in any examination procedures, which was later scaled down to a refusal to mark papers. Apologizing for offering a pay rise of only 3 per cent, the Committee of Vice-Chancellors and Principals (CVCP) insisted that universities could afford no more. After the CVCP persuaded the government to release additional funds, the offer was increased to a pay rise of 6 per cent, back-dated to April 1989, plus an extra 1 per cent to be distributed at the discretion of individual universities and a lump sum of between £150 and £285. The lecturers refused this offer.

The argument of the CVCP throughout the dispute has been that although it recognize that the offer was too low, universities simply could not afford any more. Instead of accepting the offer and claiming victory at that point, the union was determined to continue, arguing that still no money had been offered for 1988-89. Accepting an offer worth only about £50 more three months later reflects acknowledgement of members that the cost to students of prolonging the dispute would be too great.

Christine McGourty

MEDIA

Baltimore to head science journalism group

Boston

AFTER many months sitting in a "media hotseat" during the US Congress's most extensive investigation ever of scientific conduct, David Baltimore, Nobel laureate and director of the Whitehead Institute in Cambridge, Massachusetts, has taken on a new responsibility to help scientific issues receive honest coverage in the media. Last week, he was elected to chair the Scientists' Institute for Public Information (SIPI), a nonprofit organization based in New York.

Baltimore, who replaces Lewis Thomas in the position, says he has "mixed emotions" about the press handling of the continuing investigation into his research (see *Nature* 339, 163: 18 May 1989), but stresses that his experiences will have little impact on his role in the organization which seeks, in his words, "to encourage that science issues are handled" in a "good and honest" fashion by the media. He acknowledges that in the light of the investigation, the announcement of his newest title "might seem ironic", but calls the timing "sheer accident." He was, he points out, serving on SIPI's board well before he was caught

in the media spotlight.

Just last month, Baltimore admitted to feeling embattled and besieged by the congressional committee conducting the current investigation and by the press. "American science is under attack", he claimed in a written statement to a group of colleagues in Cambridge. But today Baltimore says he is confident his experience won't "in any way taint" his involvement with the media.

SIPI, now a decade old, is a small, national organization that aims to "bridge the gap between the scientific community and the press", according to spokeswoman Sheryl Burpee. Among its projects, SIPI runs the Media Resource Service, which helps journalists to locate contact sources on scientific and technical topics from the organization's large list of expert participants. Recently, the organization has also done its best to encourage more good-quality coverage of science on commercial television, something Baltimore endorses as "particularly worthwhile" because, he says, "so much of the American public gets their information" through television.

Seth Shulman

ABORTION LAW

Fetal debate continues

Washington

THE US Supreme Court is this month expected to decide a Missouri case that could either reverse or restrict an earlier decision that made abortions legal in the United States. Whatever the outcome, the abortion question will continue to colour scientific research in the United States. The Department of Health and Human Services (HHS) is at present considering extending a federal moratorium on the use of human fetal tissue for transplantation, and in Congress those who see abortion as murder have introduced legislation to regulate the "import, export or transport into or out of any State any human tissue".

Abortion is divisive issue in the United States. Hundreds of thousands of people have converged on Washington at different times this year either to endorse or condemn the Supreme Court's decision in the Roe versus Wade case which, in 1973, established the legal standing of abortion. Even the language of the abortion question is emotionally charged. People who feel abortion is morally wrong want to be called "pro-life", not anti-abortion, whereas those on other side insist they are not pro-abortion, but "pro-choice".

The federal government finds itself in an awkward position in this debate. The Justice Department has joined the state of Missouri in arguing for a change in the Supreme Court's views on abortion, and both Ronald Reagan and now George Bush have aligned themselves with the "pro-life" movement. But at the same time, HHS last year asked the National Institutes of Health (NIH) to address the issue of whether tissue from a legally performed abortion could ethically be used in research. An advisory panel of scientists and ethicists heard testimony on the promise of fetal cells for treatment of diabetes and certain nervous disorders, and on the fear that demand for fetal tissue would start a black market in baby parts.

The panel's report — which reaches the conclusion that so long as abortion is permitted by society, then using fetal tissue with the informed consent of the parents is morally acceptable — is now languishing at HHS. Also buried at the department is a movement, started last year, that would reestablish the HHS Ethics Advisory Board. The board, abandoned since 1979, is necessary before federal funds can be used for research in ethically difficult areas of reproductive medicine such as *in vitro* fertilization.

At one point late in the Reagan administration, it seemed possible that there would be an executive order banning the use of fetal tissue from induced abortion for research purposes. Although that did not happen, supporters have indicated their willingness to pursue such a

move by legislation. Representative Robert Dornan (Republican, California) has introduced the Human Fetal Tissue Transportation Act. The bill would regulate — or in the words of a member of Dornan's staff, "ban" — the interstate transportation of fetal tissue, and would force facilities storing fetal tissue to obtain a federal licence. The bill defines fetal tissue as "any matter yielded by an aborted human pregnancy and any biochemical product derived from such matter".

It is that last part of the definition that spells unexpected trouble for research. If banning transplantation of fetal tissue would slow certain areas of research, banning the use of cell lines derived from human fetuses would be "insane from the point of view of science or humanity", says Stanley Plotkin, a professor at the Wistar Institute in Philadelphia. Fetal cell lines are extremely useful in viral diagnostic laboratories, as many viruses will grow only in fetal cell lines. Fetal cells have also been used to produce a variety of human vaccines, from polio to adenovirus. Merck & Co. uses a fetal cell line to produce its rubella vaccine. If fetal cells could no longer be used, the FDA would require vaccine manufacturers to obtain new licences for producing their vaccines, a time-consuming and expensive proposition.

Not only does the federal government support research using fetal cell lines, but such cell lines are used by federal laboratories. For example, the Centers for Disease Control in Atlanta maintains stocks of nearly all identified human viruses, and these are frequently stored frozen in human fetal cell lines.

The first human fetal diploid cell line was established in 1960 by Leonard Hayflick and Paul Moorhead, then at the Wistar Institute. The line, dubbed WI-38, was obtained from a Swedish abortion of a female fetus at three-month gestational age. Hayflick, now a professor of anatomy at the University of California, San Francisco, says his work with WI-38 was the first to establish that human cells had a finite lifetime. Populations of WI-38 will divide between 40 and 60 times before the cells lose the ability to divide. This finite lifespan replaced the dogma of the time that any cell grown in culture would transform to become immortal. Hayflick says WI-38 has provided important insights on cellular ageing.

Although the Human Fetal Tissue Transportation Act has only a slim chance of passage, it nevertheless represents one end of the spectrum of concern that the abortion issue has raised, and those supporting Dornan's legislation have exhibited unflagging energy in pursuing their political agenda. **Joseph Palca**

SPACE

Galileo on its way

Washington

THE orbiter portion of the Galileo spacecraft began its trip to Jupiter last month in mundane fashion by travelling overland from the Jet Propulsion Laboratory in California, where it was built, to Florida, where it is being prepared for launch at the Kennedy Space Center. The atmospheric probe arrived in Florida earlier in the year. After about 10 weeks of testing, the two

NASA

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Preparing Galileo for launch.

parts will be joined and then set on top of the inertial upper stage booster that will propel Galileo out of Earth orbit.

The space shuttle Atlantis is scheduled to launch Galileo and its booster into Earth orbit on 12 October. Paradoxically, Galileo starts for Jupiter by heading towards the Sun for a rendezvous with Venus. It then returns for two loops around the Earth. The planetary gravity assists will help the inertial upper-stage booster to place the 2.5-tonne spacecraft on a direct trajectory. Arrival at Jupiter is scheduled for December 1995.

Joseph Palca

AIDS

Agency starts work

Paris

THE French national agency for research on AIDS (ANRS), set up last November under the joint direction of the ministries for Health and for Research, has announced the major themes it has decided to develop. It will give grants to 202 of more than 470 research proposals received. Most (118) will be for basic research, 70 for clinical and epidemiological studies and 24 for public health, human sciences and sociological studies. According to Jean-Paul Lévy, ANRS director, the FF150 million (\$22 million) budget is "realistic" for France even although it may appear small compared to funding in the United States. **Peter Coles**

Correction

About 200 French haemophiliacs have developed AIDS (see *Nature* 339, 87; 1989). □

Shake-up essential

THE policy-making procedures of the University of Cambridge, which have remained essentially unchanged since 1926, are slow, cumbersome and unsystematic, and must be altered if the university is not to lose out on research contracts and other valuable sources of income, according to an independent review group set up to examine the university's management.

Through its inability to act quickly, the university has "on more than one occasion" missed a promising opportunity to attract funds from external sources such as the research councils, says the report. It identifies the problem as a lack of delegation of authority. As a result, Regent House, the governing body, has become ineffective. Often, it is consulted about a proposal when it is already "too late to do anything but agree", says the report. When the Interdisciplinary Research Centre for superconductivity was set up, it was not possible to consult Regent House at all.

The review group recommends that the council should become the principal executive and policy-making body of the university. It also urged that the university and the colleges act in a more unified way.

The annual cost of the proposals would be £350,000. But the cost would be fully justified by the benefits that would flow from improved management of the university's resources, says the report. A university spokesman says a decision on the report is unlikely before the end of the year. C. McG.

Champagne physics

THIS year's FF100,000 (\$15,000) Moët Hennessy-Louis Vuitton 'Science for Art' prize has been awarded to Pierre-Gilles de Gennes, for his work on the physics of surfaces and interfaces. Professor at the Collège de France and director of the Ecole de Physique et Chimie in Paris, de Gennes has contributed to a wide range of disciplines including superconductors, liquid crystals and polymer physics. The prize was established to acknowledge the debt to basic science owed by manufacturers of aesthetic and luxury products. P.C.

£31 million for IRCs

THE UK Science and Engineering Research Council (SERC) is spending another £31 million on new interdisciplinary research centres (IRC), one of the most recent innovations in the British pattern of research support. As a review gets under way into the effectiveness of IRCs in supporting research, SERC says that four more IRCs are to be set up: in polymer science at the University of Leeds; in process simulation, integration and control at Imperial College, London; and in optics and laser science at the University of Southampton. A fourth, in high performance materials, will be announced soon. C. McG.

Australian code of practice

Sydney

PRESSURE from animal welfare groups and the new ethical challenges set by genetic engineering have prompted a major revision of Australia's code of practice for animal experimentation. Changes agreed last week are claimed to make the new code the most stringent and comprehensive in the world.

Guidelines on animal experimentation were laid down originally by the National Health and Medical Research Council (NH and MRC) in 1969. Since then there have been three revisions, involving the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the Australian Agricultural Association (AAC).

The new code centres on the powers given to animal ethics committees. Each institution must appoint a committee consisting of a veterinarian, a scientist involved in animal experimentation, a member of the community not involved in research (although they may also belong to the institution or university), and "a person with a strong commitment to animal welfare." While each institution is responsible for appointing its own ethics committee, the committee members must be approved by the State Government's Department of Agriculture.

At present three states, New South Wales, Victoria and South Australia, have animal protection acts that back up the federal guidelines with state law. In these states, any researcher found by an ethics committee to be contravening the code of practice could face imprisonment on top of losing grants given by federal agencies. Australia's new code is remarkable for the power given to ethics committees. Each committee issues an approval number for all experiments and researchers cannot obtain an animal without citing this number. As an added control, State Government inspectors will make surprise checks on both researchers and ethics committees.

The code contains 23 general principles. Scientists must assume that animals experience pain in similar ways to humans and experiments should only be undertaken when there is no other way to obtain the required information. Scientists must also justify the number and species of the animal required and explain why techniques that do not use animals cannot be substituted. The code emphasizes that the use of death as a routine end-point or measure should not occur in toxicological experiments or when cancer is induced, unless absolutely necessary. Ethics committees can suspend experiments and treat or kill any animals if they see fit.

The code is the result of eighteen months' work by staff of the NH and

MRC, CSIRO, AAC, the Royal Society for the Prevention of Cruelty to Animals, members of the general public, state government representatives and members of more than 500 interested parties, including animal welfare groups. Australia has seen little of the violent action of animal rights groups elsewhere (see, for example, page 407).

According to Warwick Anderson, chairperson of the NH and MRC Animal Experimentation Ethics Committee, this is because "there has been dialogue at both the State and Federal Government level. Many of the more radical animal rights groups are represented on ethics committees and are invited to attend research meetings." Despite being consulted, animal welfare groups are not entirely happy with the new code. Robyn Sullivan, President of the Humane Society and a member of the executive of the Australia and New Zealand Federation of Animal Societies says, "Our contributions have not been incorporated to the extent we would have liked".

"Post-operative pain relief will be given to experimental animals in line with veterinary practices but this is not consistent with the codes' provision for treating animals as though they experience pain like humans." A Senate inquiry into animal welfare is expected to present its findings to the Federal Government at the end of June. Convened in 1987 following pressure from animal welfare groups, the inquiry is expected to prompt the Federal Government to bring those states without appropriate animal protection laws into line with the rest of Australia.

Tania Ewing

MUSEUMS

Improving image at no extra charge

London

A THREE-month brush with a leading London-based firm of corporate image consultants has swept the halls of the British Museum (Natural History) with controversy. The museum will follow the recommendations suggested by image-makers Wolff Olins, including, at long last, an official change of name to the 'Natural History Museum' sometime this autumn. But recent newspaper reports that the museum intends to charge for expert advice to academic researchers or the public are "completely and utterly wrong", says a furious Neil Chalmers, the museum's director. The Wolff Olins report made no mention of charges, and researchers should be "reassured" that there are no plans to introduce charges for services that are now given freely. Henry Gee

ENVIRONMENTAL RELEASE

Europe avoids moratorium

Munich & Paris

THE European Parliament in Strasbourg on 25 May narrowly avoided imposing a five-year moratorium on the sale of certain products of genetic engineering. The moratorium, which was defeated by a single vote, would have applied to products of genetic engineering sold in European Community (EC) countries and which were intended for release into the environment.

The Parliament also narrowly rejected a ban on production or release of genetically engineered organisms such as bacteria altered to produce toxins. Eventually, such a ban may be reconsidered by the EC Commission, which is the executive body of the EC.

The Commission had last year asked the Parliament to respond to a set of guidelines for regulating both production and release of genetically engineered organisms. The Parliament advised slightly stricter guidelines than the Commission had suggested.

The moratorium, put forward by a coalition of Social Democrats and Greens led by West German Social Democrat Gerhard Schmid, would have applied only to products and not to scientific work. In the areas of production and laboratory work, the Parliament recommended "licensing" instead of "registration" of experiments with national authorities. The Parliament asked for risk assessment, strict liability in case of accident and public information about planned releases and their risks. Many restrictive amendments were not approved by the Parliament.

Schmid also wanted to forbid the release of any pathogenic organism and to ban any production or release involving a third category of "unacceptable" micro-organisms. The current proposal divides organisms into the categories of "high" and "low" risk. Schmid wanted an environmental impact study to be compulsory for all work involving "high-risk" organisms that could lead to products.

The Parliament accepted the Commission's view on the difficult question of how to resolve disputes between EC member states about which releases are acceptable. The Commission recommended that all scientific experiments be carried out under the auspices of individual national governments. Only when products are to be marketed in a country belonging to the EC may another member state protest. If a dispute cannot be settled between the members, it will be submitted to binding arbitration.

"A wise company" would not let it come to this, said one EC official. It would "ensure that the product was generally acceptable before marketing it in the EC".

The Parliament's proposal will now return to the European Commission for discussion and will be debated by the Council of Environment Ministers in Luxembourg on 8 June. General consent is expected, say Schmid and EC officials in Brussels. If approved, the amended bill will be given a second reading by a newly elected European Parliament later in the year.

The European Parliament has provided a forum for left-of-centre views, especially from Green and Social Democratic parties out of power in their home countries. A left-leaning coalition fought for stricter environmental regulation in this last session of Parliament before the European-wide election of a new Parliament on 18 June. Their narrow defeat on the last day of the session reflected "Christian Democrats and British Conservatives" successfully mustering their forces, according to Schmid. The moratorium was defeated by 68 votes to 67; the full contingent of 518 members was depleted by election campaigns.

Steven Dickman & Peter Coles

NUCLEAR POWER

Wackersdorf finally dies

Munich

CONSTRUCTION was halted at 5 p.m. on 31 May at the controversial nuclear fuel reprocessing facility at Wackersdorf at the urging of the West German government. It now seems that the plant, in which DM 2,600 million had already been invested, will never be completed. A minimum of DM 5,000 million is needed to finish construction. Sending nuclear fuel for reprocessing abroad will be significantly less expensive.

The government was taken by surprise by the plans of the West German utility VEBA to reprocess nuclear fuel elements at the French facility at The Hague (see *Nature* 338, 611; 1989). Now negotiations have begun to reprocess half of West German fuel at the British Nuclear Fuels Limited (BNFL) facility at Sellafield. Even at the bargain price offered by BNFL — roughly DM 1,300 per kilogram of fuel, as opposed to DM 1,500 offered by the French concern Cogema — the contract could bring as much as DM 310 million per year for 15 years to BNFL. BNFL is wholly owned by the British government.

With the death of Wackersdorf, anti-nuclear activists are losing interest in the cause. A demonstration in Munich drew only 7,500 people instead of the expected 30,000. Protest leaders said they would try to redirect the movement against the final storage of nuclear fuel and waste products in West Germany and against the French facility at The Hague. Steven Dickman

ENVIRONMENT

Massachusetts set to crush polluters

Boston

A NEW 34-member environmental 'strike force' of scientists, lawyers and undercover police officers, equipped with aerial surveillance capabilities, is the Massachusetts answer to increasing industrial pollution. Declaring that polluters have fouled water supplies in more than 100 state districts and created more than 1,000 toxic dumps, state officials, including Governor Michael Dukakis, are backing the toughest pollution counter-offensive ever seen in the United States.

Announcing the new plan, Dukakis justified the strong-arm approach by calling industrial environmental offences "violent crimes". Industrial polluters, he explained, "do violence against neighbourhoods and against the water we drink and the air we breathe".

The state's dismal financial situation

They finally nailed Big Louie in Boston on an air-pollution rap



allows no new funds for the programme which will make a start by shuffling personnel and funds from existing agencies. What it lacks in funding, however, the plan seems to make up in support from top state officials who clamoured to endorse it. Massachusetts' Attorney General promised that the new programme "will bulldoze right through the bureaucracy". And the state's secretary for environmental affairs told reporters: "The strike force is the jewel in our environmental enforcement crown".

In addition to the new environmental enforcement team, Dukakis promised legislation later this year to stiffen the state penalties for industrial polluters. But most officials say that the main problem has been lack of enforcement rather than lack of tough laws.

According to Daniel Greenbaum, head of the state department of environmental quality engineering, as many as half of those discharging pollutants into the air and water may be doing so illegally. Of those companies with environmental permits, many routinely get away with violating the terms of the regulations.

Seth Shulman

Misguided thinking on animals

SIR—Clive Hollands' chastisement (*Nature* 339, 248; 1989) of *Nature* for its publication of work involving two monkeys (*Nature* 337, 265–267; 1989) should certainly be a cause of great concern to scientists in Britain — not because of his specific criticism of the research (which is the usual list of lurid surgical details without a word about anaesthetics or scientific objectives) but because Hollands speaks with apparent authority about the British law. When he tells us that *Nature* has sinned by publishing work "which clearly would not have been authorized under British law", this is not just the wishful thinking of someone who would like to see an end to all medical research involving animals; for Hollands is a member of the Animal Procedures Committee, which advises the Home Secretary about the balance between "the legitimate requirements of science" and "the protection of animals against avoidable suffering". We who work under the strict British law had assumed that applications to perform research are considered on their merits, taking due account of the quality and importance of the science involved. Either Hollands believes that the Animals (Scientific Procedures) Act 1986 bans certain techniques *per se*, or he thinks that a single member of a large committee is entitled to pronounce in advance on what would not be acceptable. In my opinion, both assumptions are wrong and I trust that others will join me in asking the Home Secretary to comment on the right of a member of this important committee to pontificate in this way.

The research that Hollands parodies in his letter addresses fundamentally important questions about the mechanisms of development of the cerebral cortex. It was performed skilfully and humanely in one of the leading centres for biomedical research in France, which works on AIDS, Alzheimer's disease and rehabilitation after brain damage, as well as on fundamental neuroscience. I have collaborated with scientists there for many years in a study of the causes of childhood blindness and have found the animal house and laboratory facilities, as well as the quality of research, among the best that I have seen anywhere.

On 21 May, 'animal rights' terrorists broke into that very institute and stole almost 100 animals. Among them were 38 monkeys, including social family groups that had been established for nearly a decade and animals that were born in the laboratory and had never experienced any other environment. Perhaps Hollands would like to give us his opinion of the cruelty and stress involved in their forced removal and would tell us whether such treatment, without any scientific objective

at all, would be permitted under British law. I share the anger of my French colleagues at such wanton torture of animals in the name of their protection, as well as grief at the setback by years to important research.

Medical research is fighting for its survival against an upsurge of 'animal rights' activity. The response of the scientific community in the United States has been rapid and is increasingly effective. Animal rights protesters are now met with counter-demonstrations organized by medical interest groups and charities. Thirty-two Nobel laureates have recently written to the US Surgeon General urging him to warn the American public of the danger posed by the animal rights movement to legitimate and urgent biomedical research. We in Europe must also recognize the threat that we face and mobilize to resist the philistine forces that would stop the progress of medicine — from Hollands' pronouncements about his personal law to the stupidity of theft of animals and bombing of universities.

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Peer review

SIR—As editor of a national physics journal, I am sympathetic to many of the comments made by other contributors to the discussion on anonymous peer reviewing. But, for the best good of science, the first priority has to be the maintenance of high-quality well-regarded international research journals which appear regularly.

Publication of one's work (which is closely coupled to one's ego — and one's research grants) is not a democratic right; it is a privilege. The suitability of the work for publication is based on the opinion of peers, both referees and journal editors, who act as arbiters of conflicting opinions.

From a pragmatic point of view, the timely operation of science journals depends to a large extent on the willing provision of a free refereeing service, provided on invitation, by the large cadre of referees that each journal recruits. By definition, these people are also busy and productive researchers who would prefer to be doing and writing science. But they are willing to give generously, at no cost except to themselves, of their precious creative time, to read and comment on typescripts sent to them. They do this as part of their citizens' duties to the republic of science.

Human nature being what it is, I very much doubt if journal editors could easily find referees to provide this service so willingly if it were not on the implicit understanding of anonymity. Some

referees, myself included, often sign their refereeing comments, but that is as much a personal decision as that of the author in sending the paper to a particular journal for consideration for publication.

Authors, referees and editors all have their human foibles which are sometimes revealed in the anonymous referee reports. But my experience in the past few years of editing a physics journal has convinced me that only a very small minority of referees misuse anonymity to make unwarranted and discourteous remarks. Such behaviour calls for an editorial judgement on the quality of the referee's report, and the prompt securing of alternative independent assessments by others. Most of the many referees' comments that I have read relating to the few hundred papers we handle each year are helpful, supportive and often include extensive valuable suggestions for significant improvements to the quality of the papers. These comments are nearly always appreciated by the authors. An author's reputation depends on the quality of the material that actually gets into print. It can only be enhanced by supportive and helpful refereeing suggestions.

RALPH W. NICHOLLS

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SIR—Your leader on peer review (*Nature* 339, 11; 1989) interests me greatly, because I do not know just how peers are chosen these days. In particular, although those who are concerned with fields contiguous to the work described may be able to read papers referred to them without difficulty, their very proximity to the matter may on occasion prove to be a disadvantage.

The current plethora of publications is the inevitable consequence of the view in academic circles that one's status is a function of the number of one's personal publications. This has changed the academic purpose from that of ascertaining natural laws to the aggrandisement of individuals: once the individual becomes more important than the community in which he lives and works, the end of that community is in sight.

Incidentally, the 'black-balling' system still operates. At least one technical society I know allows membership by invitation only: a friend of mine who had to move to a different part of this country and was concerned to get to know local people enquired of the local branch of a famous international body about joining it, and was told to wait until he was asked. Whether one would wish to join anything which behaved in this way, is a question that borders on the moot.

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New light on the Lysenko era

Valery N. Soyfer

Previously unpublished material shows how Trofim Lysenko both deceived and won the support of Stalin, and destroyed genetics in the Soviet Union.

TROFIM Denisovich Lysenko's life and work have been much analysed and discussed in the world's literature. It is well-known that his activities were among the factors leading to the breakdown of biological, agricultural and, to some extent, medical science in the Soviet Union. They contributed to the transformation of Russia from a country that exported grain and other agricultural products to the whole world into an importer of grain, butter and meat. So the study of the life and activity of Lysenko may illuminate the flaws which, even now, cripple Soviet agriculture — and Soviet science.

Another reason for returning to the history of lysenkoism is that much has so far been unclear and even mysterious¹. In particular, there is much to be learned from the relationship between Lysenko and Vavilov in 1930–35 and Lysenko and Stalin in 1947–48.

The question of whether Lysenko's earliest works were independent and original contributions to science has often been asked^{2,3}. It is generally accepted that they were original and pioneering in character. More recently, Røll-Hansen pointed out how widely this idea is held by remarking⁴ that "some of his [Lysenko's] physiological work was highly praised even by his strongest critics among the geneticists". So how independent of previous researchers was Lysenko in his early work?

Lysenko published two articles of little significance as early as 1923, when working at a small plant-breeding station in the town of Belaya Tserkov' in the Ukraine⁵. In 1925, while still at Belaya Tserkov', Lysenko graduated by correspondence from the Kiev Agricultural Institute, but, once he had received his agronomist's diploma, he unexpectedly left for the Caucasus (Azerbaijan), where he found a job as a junior specialist on the selection of legumes, at an experimental station in the town of Gandzha (now Kirovobad). The director of the station, the well-known Russian agronomist N. F. Derevitsky, set Lysenko a problem: to try to acclimatize beans for Azerbaijan as a green-manure crop. In the autumn of 1925, Lysenko for the first time sowed peas as a winter crop. Thanks to the mild winter of 1925–26, they yielded a good vegetative mass. This result was promising. The work should have been repeated on a wider scale, to determine appropriate dates for autumn sowing, to choose the best cultures, to

work out the agrotechnical details and to settle on the most appropriate varieties. But at this point, an event occurred that drew Lysenko away from this work and directed his activity into a very different field.

To understand this event, it is important that, from the point at which Soviet power was established in Russia, Lenin and, after him, Stalin did everything possible to train cadres of a new intelligentsia, intended to replace the specialists trained under the tsarist regime. Since the new leaders adhered to the class approach and considered that the so-called "bourgeois specialists" were for the most part hidden enemies of the new order, there was a drive to train persons of worker or peasant origin (the so-called "red intelligentsia"). All paths were opened to members of these classes; special attention was paid to bringing them forward.

Trofim Lysenko satisfied the new criteria perfectly. He had been born in 1898 into a peasant family, and was late in learning to read and write; only at thirteen did he enter a two-form village school. Afterwards, he attended the Poltava Lower Horticultural School for rather more than two years. Only in 1917 did he enter the Uman' Horticultural School, where he was a student for about four years.

The course of Lysenko's studies was far from normal. Between 1917 and 1920, Uman' was in the battle zone between warring armies and changed hands several

times between the Whites, the Greens (Ukrainian peasant insurgents) and the Reds. Taking advantage of his peasant origin, Lysenko then enrolled at the Kiev Agricultural Institute, although he studied there externally and, as a result, was not able to attend regular higher education courses. But the circumstance that he graduated not from a bourgeois, but from a Soviet, higher-education institution ensured that he continued to benefit from all the advantages guaranteed by the Soviet leadership to persons of peasant origin.

So it is hardly surprising that when, in 1927, the well-known Moscow journalist V. Fedorovich visited the Gandzha experimental station, he was presented to the budding agronomist Lysenko. In his article in *Pravda* on 7 August 1927, Fedorovich lauded Lysenko's first experiment with the peas as a substantial discovery, and announced that Lysenko (whom he hailed as a "barefoot professor") had made an outstanding discovery that would allow the peasants of Azerbaijan to avoid all future difficulties due to shortage of fodder. (As it happens, green-manure crops are not cultivated on any scale in Azerbaijan.)

Lysenko's first work thus remained unfinished; no publications other than Fedorovich's article mentioned it. But Lysenko himself considered that the matter had been dealt with and switched to new work. Quick as lightning, he "solved" another problem, namely the



Lysenko, newly appointed president of the Soviet Academy of Agricultural Sciences, surrounded by colleagues at Odessa.

effect of low temperature on plants.

From the autumn of 1926 to the spring of 1927, several of Lysenko's trainees collected data on the germination, development and fruiting of cereals (wheat, rye, oats and barley) and cotton. In Russia at that time, the same problem was being studied by Vavilov's collaborators N. A. Maximov and G. S. Zaitsev⁶. Lysenko's data were presented in long monotonous tables which he furnished with primitive commentaries. They were published in 1928 as a booklet titled *Proceedings of the Gandzha Experimental Station*⁷. Instead of working out his results statistically and presenting the data in summarized form, he devoted 110 of the 169 pages in the book to tables with raw data.

Lysenko finally set out his nine-point conclusion, which can be reduced to the single observation that, for the initial phases of plant development to be completed, definite quantities of heat are required. The method he used was close, even in its detail, to that developed by the talented Russian physiologist G. S. Zaitsev⁶ who studied the effects of various environmental factors, not simply temperature, on the phases of development of cotton.

It is relevant and important that, in January 1929, Lysenko attended the First All-Union Congress on Genetics, plant breeding, seed growing and pedigree stock rearing in Leningrad. There he announced that, on the basis of his work, he could recommend the cold treatment of winter-wheat germinants so that it would be possible to sow winter instead of spring wheat⁸. But in the discussion, his proposal that cold germination (he called it 'vernalization') should be put into practice immediately was criticized by Maximov.

Despite the criticisms and the fact that his method had not been tested scientifically, Lysenko managed, in the summer of the same year, to organize a noisy press campaign in favour of his 'vernalization'^{9,10}. The agricultural bosses were captivated by the novelty¹⁰ and, in the winter, the official Party organ *Sel'skokhozyaistvennaya Gazeta* (*Agricultural Gazette*) launched a discussion of Lysenko's ideas. At that stage an article by the outstanding Soviet scientist N. M. Tulaikov stated¹¹:

At the beginning of 1927 [at the inception of Lysenko's work on vernalization] at the experimental station at Gandzha, I happened to have a number of discussions with Lysenko ... who was working on the application to various plants of the region of the laws established by Professor G.S. Zaitsev on the sums of the temperatures necessary for the various phases of development of cotton.

Tulaikov wrote that by then, Lysenko had "already outlined ... a certain rule", but the reference to the fact that Lysenko knew Zaitsev's results is quite definite.



Lysenko offers herbarium samples as proof of his claimed transformation of wheat into rye (1948).

Indeed, Lysenko himself, in a book published in 1928, mentioned Zaitsev at three crucial points, comparing Zaitsev's results with his own and acknowledging that they were similar (see ref. 7, pages 16, 136, 142). But all references of this kind afterwards disappeared from Lysenko's works, and he began to insist on his own unconditional priority in the matter. Apparently the chief reason for this behaviour was that, in January 1929, while travelling from Central Asia to Leningrad to the First All-Union Congress on Genetics and Plant Breeding at which Lysenko first put forward his hypothesis, Zaitsev became ill and died in Moscow without reaching Leningrad. Thereafter, Lysenko decided never to mention his precursor again.

The sources of Lysenko's first work have been forgotten with time. In 1937, N. M. Tulaikov, the only living witness of Lysenko's use of Zaitsev's work, was accused of hostile activity. (The charges were given by Lysenko himself in an article published in *Sotsialisticheskoe zemledelie* (*Socialist agriculture*) on 4 April 1934; a week later, his close collaborator V. N. Stoletov published in *Pravda* an enormous article accusing Tulaikov of wrecking.) Soon afterwards, Tulaikov was shot.

M. A. Popovsky in the Soviet Union¹² and D. Joravsky in the West¹³ were the first to point out that N. Vavilov was Lysenko's scientific patron. The most authoritative Western biography of Vavilov supports this view¹⁴. Popovsky cited extracts from Vavilov's statements proposing Lysenko for membership of the Ukrainian Academy of Sciences, for the Lenin Prize and as a corresponding member of the USSR Academy of Sciences.

But Medvedev¹⁵ categorically denies that Vavilov was the one who mainly advanced Lysenko in scientific circles, saying that, as an important administrator, Vavilov might have signed the documents in support of Lysenko without having read

them. But the facts unearthed by Popovsky contradict that. Moreover, I have been able to find many previously unknown statements of Vavilov that confirm that it was Vavilov who played the chief role in Lysenko's scientific advancement¹.

Why did Vavilov succumb to this fatal blindness and not recognize Lysenko as a dangerous upstart from the outset? Popovsky takes the view that the explanation lies in the social conditions of the times, and specifically in the demand of the Party of Bolsheviks that people "from the furrow and the bench" should be advanced in all spheres of society, science included. As Vavilov sincerely accepted the revolution, putting himself at its service, he would have acknowledged this "social imperative" and would have seen Lysenko as an obvious candidate to be "promoted from the people".

The logic is tempting, but the proposition only partly explains Vavilov's motives. A more likely explanation lies in the nature of the field in which he worked. In 1925, he had become director of the Institute of Applied Botany and New Crops (later the All-Union Institute of Plant Industry) and had begun to assemble a collection of seeds of wild and cultivated plants from all over the world. The motives for collecting this "gene-bank of world flora" were not exclusively scientific; Vavilov intended to make extensive use of the seeds collected so as to increase the rate at which new varieties of cultivatable crops were introduced. This would be done by the transfer of valuable genes to strains extant in the Soviet Union. Not himself an expert on plant breeding and without much experience of fieldwork, Vavilov could only hope the plan was feasible.

In practice, Vavilov and his colleagues encountered problems that were not easily soluble. The physiological requirements of the seeds collected from elsewhere were different from those of the local varieties. They germinated,

sprouted, flowered and fruited in different ways, and many would not germinate at all in the Russian climate. Crossing the "foreign" with the local varieties proved a practical impossibility in most cases. And then, suddenly, there appeared a man who confidently declared that he could solve a far more complex problem — the conversion of winter into spring crops by vernalization (cold treatment).

Lysenko was then working at the Gandzha station, a part of Vavilov's institute, so that it is not surprising that the director soon learned of his work. This apparently explains why Lysenko, whom nobody had heard of, suddenly received the prestigious invitation to take part in the First All-Union Congress on Genetics and Plant Breeding that Vavilov himself had organized. Vavilov was apparently convinced that if the complex transformation of winter to spring crops were feasible, the problem of synchronizing germination and flowering of other plants might be easily solved.

This interpretation is confirmed, in particular, by the fact that, several days before the meeting, Vavilov gave an interview to the Leningrad newspaper *Smena* in which he spoke highly of the idea of transformation¹⁶. It was also especially important for Lysenko's later career that Vavilov put the utmost value on the experiment for the vernalization of winter wheat, calling Lysenko an outstanding scientist at several sessions in 1930 and 1931 of the Presidium of the Academy of Agricultural Sciences and of the Collegium of the State Commissariat for Agriculture of the Soviet Union¹⁷. Thus in September, 1931, at a meeting of the State Commissariat for Agriculture, Vavilov said:

Of especial interest ... is the work of Lysenko, who has actually managed in practice to change late-ripening into early-ripening strains and to convert winter into spring varieties. The facts which he has established are indisputable and are of considerable interest ... Lysenko's experiments show that, with special pre-sowing treatment, late Mediterranean varieties of wheat may be converted into early varieties in our conditions. Many of these varieties surpass our ordinary varieties in quality and productivity ... Rapid organizational collective persistent work is required in order to realize the most interesting facts established by Lysenko¹⁸.

My case is further strengthened by the fact that Vavilov, delighted at the possibility of synchronizing the flowering of different plants, gave instructions for the reserve of wheat at the Institute of Plant Industry to be vernalized and sown near Leningrad and Odessa. (From 1929, Lysenko worked at the Odessa Institute of Genetics and Plant Breeding.) A proportion of the plants of the varieties that did not normally yield ears in the Odessa region gave ripe ears. Lysenko promptly exaggerated

this result and presented it as proof that all varieties could then be sown in regions that were abnormal for them. This categorical conclusion greatly delighted Vavilov who, taking it literally, on many occasions hailed the method of vernalization. For example, in 1934, in a paper at a conference on planning genetic-selection work, Vavilov said:

Perhaps in no other division of plant physiology have there been such profound advances as in this field ... In this respect, we consider the work of T. D. Lysenko to be outstanding.

The relatively simple procedure of vernalization and the possibility of its broad application are opening up broad horizons. Investigations of a world-wide range of wheat and other crops under the action of vernalization have revealed facts of exceptional significance. The world-wide range of wheat varieties has been entirely transformed by this simple procedure¹⁹.

But there had been no *complete* alteration of the worldwide range. What had happened was entirely different — the general acceptance of Lysenko's inflated estimation of his own work. Even in 1935, after five years of trials of vernalization by scientific institutions, scattered over different regions of the vast Soviet Union (vernalization was tested on 35 varieties of wheat), the head of the trials, Academician P. N. Konstantinov, came to this conclusion:

On average, over the years, what we have observed is that there is here a decrease and there an increase due to vernalization, but on the average, over five years, vernalization has given almost no increment of yield²⁰.

Vavilov nevertheless continued to place a high value on Lysenko's work. For example, on 17 June 1935, at a meeting of the Presidium of the Academy of Agricultural Sciences, Vavilov said²¹, "Lysenko is a careful and highly talented researcher. His experiments are irreproachable". Similarly in 1935, he took part in two meetings in the Kremlin at which Stalin was present and put an overwhelmingly high value on Lysenko's contribution to science and agricultural production²².

Only in 1936–37 did Vavilov realize what sort of a person his protégé really was. He began an active campaign against Lysenko, but by then it was too late. The

"people's scientist" had become Stalin's pet, had acquired enormous power and had begun the methodical annihilation of his scientific opponents. Lysenko had declared war not for the life but for the death of genetics, and of the study of plant hormones and other biological disciplines based on the principles of genetics. He was preaching the doctrine of the inheritability of acquired characteristics. Lysenko even launched an attack on Vavilov himself. As early as 1935, in Stalin's presence, he publicly named Vavilov as an enemy of his own "science". These attacks ended in tragedy; on 6 August 1940, Vavilov was arrested and in 1943 he died of exhaustion in Saratov prison.

The Second World War led to the destruction of agriculture in much of the European territory of the USSR, with the result that the ineffective system of collectivized agriculture could not cope with the ever-increasing difficulties. Worse, in 1946, the Soviet Union suffered enormous crop-failures across all its agricultural zones — Russia in Europe, the Ukraine, Siberia and Kazakhstan. The famine of 1947 is still remembered in the Soviet Union.

Because practically all the major leaders of agriculture had been arrested in the late 1930s, Lysenko became the autocratic 'boss' in biology and agronomy in the Soviet Union. In famine conditions, the government and Party required from him scientific projects that would allow immediate relief from the problems of food and fodder shortage.

But Lysenko was unable to propose anything realistic, and his former promises to produce a sharp increase in yield by vernalization, summer planting of potatoes, intra-strain crossing, changing the selection criteria and the rapid introduction of new varieties were ineffectual. So too were his promises to introduce, in between one and two years, new varieties of wheat for the Ukraine and Siberia, to introduce new strains of cotton, to destroy insect predators by "pasturing" poultry in the fields (the poultry were supposed to peck up the grubs and caterpillars of the insects) and other similar proposals.

Lysenko had also harmed his own cause



Lysenko (right) with party general secretary Nikita Khrushchev and M. Suslov (left) at an experimental farm in 1955.

by his resolute repudiation of plant hormones, and his reputation was undermined when the Dane Vent and the Soviet physiologist N. G. Kholodny were honoured for their discovery.

At the personal level, Lysenko suffered much unpleasantness as a result of his brother's decision to go over to the Nazis and then to defect to the West. In the Soviet Union, the relatives of those who left the country were considered traitors. (There was a special article in the Criminal Code to that effect.)

Lysenko's relationship with the authorities was also affected, immediately after the war, by the degree to which Western society was shocked by the fate of N. I. Vavilov, G. D. Karpechenko, N. V. Timoféeff-Ressovsky and other outstanding scientists whom Lysenko and lysenkoists had publicly attacked and who had then mysteriously disappeared. At that time, British and American leaders, in particular Churchill, made repeated personal appeals to Stalin asking what had happened to Vavilov (Yurii Zhdanov, personal communication, 22 December 1987). Stalin knew, of course, that this outstanding Soviet scientist had been arrested precisely because he had opposed Lysenko. Finally, it is also relevant that, in 1947-48, the major Soviet biologists who were still alive openly declared themselves against Lysenko's new ideas, particularly his assertion that he had found serious errors in the work of Darwin. According to Lysenko, there is no intra-species struggle in nature, but mutual cooperation between plants of a single species.

Lysenko's dethronement was further assisted by Yurii Zhdanov, head of the Science Section of the Central Committee of the Party and son of the influential Secretary of the Central Committee of the Party, A. A. Zhdanov. The younger Zhdanov was openly sympathetic to the geneticists, expressing his scepticism about Lysenko. Among the many letters from geneticists, plant breeders and agronomists, criticizing Lysenko that reached the Central Committee was a long manuscript by Vladimir Efroimson which set out in concise form the details of Lysenko's scientific and organizational errors.

The post-war change of attitude towards Lysenko was reflected in the following events:

■ In 1945, A. R. Zhebrak who, after the arrest of Vavilov and the other plant geneticists, had become the most important specialist in this field, and who also worked in the office of the Party Central Committee apparatus, published an article called "Soviet biology" in the journal *Science*²³. This amounted to a careful criticism of Lysenko. That there should be an article had been suggested to Zhebrak by the then Politburo member A. N. Voznesenskii, but Zhebrak obtained permission for

the project from another member of the Politburo of the Central Committee of the All-Union Communist Party (Bolsheviks), A. S. Sherbakov, head of the Sovinformburo (A. R. Zhebrak, personal communication).

■ Steps were taken to set up a new Institute of Genetics and Cytology within the framework of the Soviet Academy of Sciences, as an alternative to Lysenko's institute. The new president of the Academy, Sergei Vavilov, brother of the Nikolai Vavilov who had perished in Stalin's torture chambers and who had been elected president of the academy on 17 July 1945, helped²⁴ in this project. The project progressed quite a long way — it was approved by the Central Committee, the appropriate documents were forwarded to the Soviet government, the recruitment of staff began and a source of funds was opened up.

■ Some of the new members elected to the Soviet Academy of Sciences in 1946 were mostly people who had openly expressed their disagreement with Lysenko. There was even a geneticist among them. Furthermore, in 1946, Stalin prizes were awarded to two specialists who were well-known for their negative views of Lysenko: V. S. Nemchinov, for his work *Agricultural Statistics*, and V. I. Edel'shtein for his textbook *Vegetable Growing*.

■ On 9 February 1947, at a plenary session of the Central Committee devoted to agriculture, Lysenko's taboo on hybrid maize was revoked, his proposal to replace winter by spring wheat in the Ukraine was reassessed as "incorrect" and it was noted that there were no good varieties of winter wheat for Siberia (although Lysenko had been trumpeting that such varieties had either been created already, or else were in the process of being created by him personally).

■ According to Zhores Medvedev²⁵, A. A. Zhdanov went to the Organizing Bureau of the Central Committee with a proposal that the leadership of the Academy of Agricultural Sciences should be strengthened. This, in bureaucratic language, was a proposal to find someone to replace Lysenko as president of Lenin's Academy of Agricultural Sciences.

■ In 1947 and 1948, scientific conferences at Moscow University were attended by enormous audiences (up to 1,000 people at the last of them) at which Lysenko's views on the reform of darwinism were subjected to searching criticism.

■ The final chord of the whole anti-Lysenko symphony was a speech by Yurii Zhdanov, head of the Division of Science of the Central Committee, at a seminar of district committee and provincial committee lecturers in Moscow. Zhdanov's lecture was almost entirely devoted to criticism of Lysenko²⁷. Zhdanov said that Lysenko "to a great extent is struggling

against shadows of the past", and that, based on his

very narrow-minded conceptions, he definitely delayed the usage of new hybrid corn in our country. I consider that in this example we have new theoretical narrow-mindedness overgrowing into definite material detriment. And we are compelled to consider critically one or another new supposition proposed by a school of Lysenko.

Zhdanov spoke about the failures of

... the attempts by Lysenko to 'hunt out' new plant varieties for 2 or 3 years, as well as his promise to select for Siberia winter wheat which will be resistant to frost and which will not differ in their stability from domestic varieties.

Zhdanov characterized Lysenko's claims as "undesirable accusations" which brought harm to Soviet agriculture. One of the most important requirements of the lecture was the denial of Lysenko's favourite thesis: the wish to divide the Soviet scientists into bourgeois and socialist specialists.

It is wrong to think [Zhdanov said] that there is a struggle between two bourgeois schools, one of which represents Soviet views and another bourgeois Darwinism. I think that such a contradiction should be rejected, because discussion takes place between the scientific schools, both of which belong to Soviet science, and neither of these schools can one call as bourgeois.

The final sentence of this lecture was as follows:

It is necessary to liquidate the attempts to establish the monopoly in one or another division of science, because every monopoly leads to stagnation... Trofim Denisovich tried to do many things, and we say to him: Trofim Denisovich, you also did not do many important things. Moreover, you close your eyes on the whole set of new forms and methods of the reorganization of nature.

Such lectures were then of exceptional significance, for they brought to the attention of those responsible for the current Party line the latest resolutions of the leadership, as well as providing ideological directions for the immediate future. Zhdanov's sharp criticism was taken as clear evidence of the inevitable and imminent downfall of Lysenko²⁵.

Immediately after this lecture, Lysenko decided on a desperate step. Once again, he showed that he was by no means a coward and was an excellent psychologist. Lysenko wrote to Stalin and to the senior Zhdanov saying that he was willing to give up the presidency of the Academy of Agricultural Sciences, not because there were fatal errors in his work, but because all his life geneticists and other opponents of his science ("Michurinist science") had prevented his proposals from being put into practice, had slandered him and were now thriving to such an extent that they had even persuaded Yurii Zhdanov to support them.

Lysenko plainly planned his reply to

Zhdanov's criticism carefully. He wasted few words on the principal offender, Yurii Zhdanov. The chief emphasis in his letter is his undisguised request to be allowed to deal with those who held dissenting views. That was completely in Stalin's style.

Lysenko's letter embodies a double distortion of the truth. He represents himself as an unfortunate little lamb against whom the reactionary geneticist-wolves are sharpening their fangs. (This, of course, was a blatant lie; the geneticists were still reeling from the arrest of many of their leaders, the death of N. K. Kol'tsov and, after the 1936 and 1939 discussions, the general weakening of their position.) But Lysenko also sought to convey the impression that, because of this situation, it was difficult for him to "press forward" with the application of his proposals.

The self-pitying tone of the letter is deliberate. His self-abnegation was calculated to demonstrate his humility and his readiness to carry out instructions unquestioningly. This, he would have known, would have been the safest way of obtaining *carte blanche* to deal with potential and declared enemies.

Significantly, Lysenko did not conceal his intention of dealing with those with dissenting views. That is the relevance of the concatenation of two of his complaints. First, he said, he had been "accused more than once" of having applied "administrative blocks" in the interests of the "Michurinist direction to which [he] subscribe(d)" to the "other, contrary, direction". But then, he continued, "for reasons outside [his] control, this, unfortunately, was far from being the case".

Lysenko's suggestion was that he was not in a position to launch a pogrom against his opponents. His calculation was that the leaders of the Central Committee, Stalin and Zhdanov, who had a propensity for crushing their opponents, would not fail him. He was seeking their agreement for a pogrom in genetics. He understood quite well how to approach Stalin and Zhdanov senior.

Although Yurii Zhdanov's lecture created a considerable stir, it led to no real unpleasantness for Lysenko. But, equally, there was no immediate response to his letter to Stalin and Andrei Zhdanov. So Lysenko resolved on a second course of action. Through the Minister of Agriculture of the USSR, I. A. Benediktov, he obtained a transcript of Yurii Zhdanov's speech. On 11 May 1948, he returned it to Benediktov, attaching yet another note which ended with him suggesting that he should resign as President of the Academy of Agricultural Sciences²⁵.

Lysenko began by saying that the transcript had been "somewhat toned down" compared with what he had heard, and continued:

Lysenko's appeal to Josef Stalin

To the Chairman of the Council of Ministers of the Union of SSR, Comrade Josef Vissarionovich STALIN "To the Secretary of the Central Committee of the VKPV, Comrade Andrei Aleksandrovich ZHDANOV"
from Academician T. D. Lysenko

It has become very difficult for me to go on working, both as President of the Lenin All-Union Academy of Agricultural Sciences and even as a scientist. Therefore, I have decided to turn to you for help. A most abnormal situation has arisen in agrobiological science.

That there has been, in this science, and still continues a struggle between the old metaphysical line and the new Michurinist line is well-known; that I consider normal.

At present, for obvious reasons, the Weissmanists and neo-Darwinists have adopted a new strategem. Without changing anything in their science, they have proclaimed themselves to be supporters of Michurin and have accused us, who subscribe to Michurinist science and who are developing it, of having restricted and perverted that science. It is also obvious why all this pressure from the Weissmanists and neo-Darwinists is aimed first and foremost at me personally.

Under these conditions, it is extremely difficult for me, as head of the Academy, to go on working.

Up to a point, all this has seemed to me normal and understandable. The test of the validity of the objectives and methods of scientific work in our country is the degree to which they assist socialist agricultural practice. This was the principle from which I, as head, drew the strength to develop Michurinist science and to assist practice as much as possible. This was also the best means of combatting metaphysical arguments in biology.

In spite of the lack of scientific objectivity and, often, the downright slanders to which the opponents of the Michurinist persuasion have resorted, and although it has been difficult for me, drawing inspiration from collective and state-farm practice, I have found the strength in myself to resist the pressure and to go on improving my work in theory and practice.

But now a situation has arisen which has really made me lose heart. On 10 April this year, the head of the Science Division of the Propaganda Section of the Central Committee of the All-Union Communist Party (bolsheviks), Comrade Yurii Andreevich Zhdanov, delivered a lecture at a seminar of Party provincial committee lecturers on the subject "Debatable questions in contemporary Darwinism".

In this lecture, the lecturer poured out against me personally, by name, the false accusations of my anti-Michurinist

opponents. It is clear to me that the allegations put forward by this lecturer — the Head of the Science Division of the Propaganda Section of the Central Committee of the All-Union Communist Party (bolsheviks) — were accepted as the truth by the large audience of Party regional committee lecturers. So the falsehoods perpetrated by the anti-Michurinists and neo-Darwinists will have even greater effect in the provinces, both among scientific workers and among agronomists and leaders of agricultural practice. And for the scientists whom I lead, the road to the practical application of their results will be beset with great difficulties. This has come as a great shock to me; it is difficult for me to endure it.

Thus I am turning to you with a request which is very important for me: if you think it appropriate, give me your help in this matter, which seems to me to be of no small importance for our work in agricultural and biological science.

It is untrue to say that I cannot take criticism. This is so far from the truth that, in the present case, I will not discuss it in detail. I have always submitted all my work, both theoretical and practical, to criticism; from that, I have learned to draw what is useful for the cause, for science. All my scientific life has been conducted under criticism, which has been a good thing.

The lecturer had never sent for me, and had never spoken to me personally, although the whole criticism of his lecture was basically aimed at me. I was refused a ticket for the lecture and I listened to it carefully, not in the auditorium, but in another room, on an extension-speaker in the office of Comrade Mitin, the Deputy President of the All-Union Society for the Propagation of Political and Scientific Knowledge.

The essence of the lecture, in my understanding, may be judged even from the very fragmentary notes I made of the concluding part of the lecture. These I enclose separately.

I have been accused more than once of putting — in the interests of the Michurinist persuasion in science to which I subscribe — administrative impediments in the way of the other opposite persuasion. But in fact, for reasons outside my control, this, unfortunately, is far from being the case. The constraints that have been imposed have been on the persuasion

which I support, the Michurinist persuasion.

I do not believe it is an overstatement if I say that personally, as a scientific worker and not as president of the Academy of Agricultural Sciences, I have by my own scientific and practical work given no little assistance to the growth and development of Michurinist science.

The principal sorrow and difficulty of my work as president stems from an obligation placed on me which I am profoundly convinced is incorrect — to ensure the development of different persuasions in science. (It is not a matter of different branches of science, but specifically of different persuasions.)

For me, this obligation cannot be undertaken. But I have not been able to block the opposing view, first because such questions in science are not solved by administrative means and, second, because the neo-Darwinist defence has been so strong that I could not have blocked it.

In reality, I have not been the president of the Academy of Agricultural Sciences, but the lone defender and head of the Michurinist persuasion, which is still in a clear minority in higher scientific circles.

The difficulty has been that, as president of the Academy, I have had to present the scientific and practical work of the representatives of the Michurinist persuasion (a clear minority in the Academy) as the work of the entire Academy. But the anti-Michurinists have not spent their time on creative work as much as on random attacks and calumnies.

I can assist the development of the most diverse branches of agricultural science, but only within the context of the Michurinist persuasion, that which acknowledges the transformation of living nature arising from the conditions of life and which acknowledges the inheritance of acquired characteristics.

Long ago I accepted and I now subscribe to and am developing V.I. Yam's studies on agriculture and Michurin's studies on the development of organisms. Both these studies belong to a single persuasion.

I should be happy if you could find it possible to provide me with the opportunity of working only in this field. Here I sense is my strength and I would be able to make a useful contribution to our Soviet science, to the Ministry of Agriculture and to our Collective-and-State farm experience in its different fields.

Forgive my clumsy style. It is due largely to my current situation.

ACADEMICIAN T. D. LYSENKO
President of the Lenin All-Union
Academy of Agricultural Sciences
17 April, 1948

...the lecturer presents as from him personally the old calumnies of the anti Michurinists-Morganists-neo-Darwinists.

This criticism was made in secret from me, so that I would not be able to object and refute it either verbally or in print.

In the corrected transcript, no references are made to the titles of my works, nor to the pages from which the quotations are taken. Hence the reader cannot compare what the lecturer said on each question with what I said. I have already stated more than once that, in these conditions, into which I have been thrust, it is impossible for me to work as President of the Lenin All-Union Academy of Agricultural Sciences.

For the good of agricultural science and its application I seek to raise the question of my release from the post of President and to be given the opportunity of carrying out scientific work. I should then be able to accomplish considerably more to the benefit both of our agriculture and for the development of biological science of the Michurinist persuasion in various branches,

including the training of scientific workers.

The tone of this letter is quite different, from which one may cautiously conclude that, by then, Lysenko had some information of Stalin's reaction to his first letter, and that he wished to speed up events. His categorical accusation of Yurii Zhdanov as incompetent and even unscrupulous is a sign of that.

There were good reasons for hurrying events. The Academy of Agricultural Sciences needed to fill several vacancies left by those of its members who had perished in prisons and labour-camps as well as those who had simply died naturally. These were to be the first elections in the history of the Academy of Agricultural Sciences, and many of Lysenko's scientific opponents had been nominated unopposed. If they were elected, Lysenko would certainly lose control over the Academy of Agricultural Sciences.

Two years earlier, Stalin had summoned Lysenko and given him a little bag containing the seeds of an unusual branchy-eared wheat, asking him to consider whether the wheat could be improved. The burst of attention paid to this wheat in the Soviet Union, and Stalin's own sudden interest in it, were typical of that time. Once again, there gleamed the prospect of solving the complex and urgent problem of supplying the population with food simply and cheaply — by introducing just one miracle-variety.

Interest in branching wheat had flared up even before the Second World War, when the news was carried all over the country that a simple woman working on a collective farm in Central Asia, Muslima Begieva, had obtained a wondrous harvest by sowing branching wheat. But after the war, according to Begieva herself, things had gone wrong, and the wheat had stopped branching. However, to make up for this, in Georgia, Stalin's own birthplace, the seeds of this wheat had, it was said, been renewed and improved.

The moment at which Lysenko received from Stalin's own hands the packet of seeds of branched wheat is crucial for the understanding of Lysenko's personality. When Lysenko heard Stalin's wish that branched wheat should be improved, he ought to have refused the request; he already knew perfectly well that this was an impossible task. Stalin, naturally, did not know what a real expert on wheat, as Lysenko always made himself out to be, would have known — that interest in branching forms went back more than a century, both in Russia and abroad.

The Russian scientists N. Shcheglov (1828), M. Spafareiev (1837) and many others had described this wheat in detail and had shown that it could not be used to increase the yield per unit area, because its plants would develop normally only if they were sown further apart. So great an authority on wheat as Professor A.M. Bazhanov wrote in 1856 that, "multi-eared wheat, like a pampered child, requires, in addition to rich soil, well-spaced sowing. It cannot endure even slight frost and suffers more than other simple kinds from smut and rust. Although each individual ear yields more grains, compared to the ears of simple grains, when one considers the total yield of all the ears for a known area of land, it is always found that multi-ear wheat has no advantage over the ordinary kinds"²⁶.

Lysenko, of course, most probably did not know this. He was not conspicuous for his erudition, and had failed to read not only earlier but even much later works. Nor had he paid attention to a number of publications by the pupils of Vavilov during the 1930s. But nevertheless, there are data to show that Lysenko knew quite well that the advantages of branched wheat

were purely superficial.

First, the journal *Yarovizatsiya* (*Vernalization*), which Lysenko himself edited, had published in 1940 an article on the properties of branching forms²⁷. Second, in 1938 the newspaper *Sotsialisticheskoe zemledelie* (*Socialist Agriculture*) had published a photograph of Denis Lysenko, Trofim's father, holding ears of branching wheat in his hand. The caption made it quite clear that the two Lysenkos, father and son, had set their sights on this wheat, and had studied its properties, but understood that nothing useful would come out of it.

Yet when Stalin approached Lysenko, his affairs were in such a bad state that he had nowhere else to turn, and he decided to resort to bluff. So as not to have to refuse straight out to carry out the task, he promised Stalin that he would start working on branched wheat. When the earth began to shake under his feet, he turned to direct deception and informed Stalin that the seeds he had been handed two years earlier had been improved so much that, in the coming year, the production of grain would be increased fivefold.

This promise gave Stalin grounds for putting an end to Lysenko's downfall. Only recently I have learned that, in May 1948, Stalin urgently summoned the whole staff of the Politburo of the Central Committee and in very sharp form criticized the organizers of Yurii Zhdanov's lecture (D. T. Shepilov, basically) and Zhdanov himself (Y. A. Zhdanov, personal communication, 24 December 1987). Stalin repeated again and again one phrase: "Who dared to offend such a good person!" (D. T. Shepilov, personal communication, 8 January 1988). In July, 1948, the innovator was summoned to an audience with Stalin, during which Lysenko persuaded the leader that the work with branched wheat was almost concluded, and that it remained only to produce more stocks of the variety before distributing it into the boundless fields of

Russia. Lysenko sought and obtained permission to name the new varieties "Stalin branching".

At the same time, Lysenko also won Stalin's consent for the final administrative elimination of all his critics, for a final ban on genetics in the USSR and for the cancellation of the elections in the Academy of Agricultural Sciences. Instead of the elections, Stalin simply co-opted 35 new members to the academy, most of them supporters of Lysenko. The appointments were announced in *Pravda* on 28 July 1948.

Among the new members were S. N. Muromtsev, an officer of the KGB (NKVD) who had previously been head of the special prison for arrested scientists. He took his job so seriously that he personally took part in the beating of two professors, Zdrodovsky and Zilber. Two days later saw the beginning of the August 1948 session of the academy, which destroyed genetics in the Soviet Union.

A week later, *Pravda* published a letter from Yurii Zhdanov to Stalin renouncing his earlier position and saying his errors were due to political immaturity and a tendency to substitute "scientific objectivity" for "Party principledness", which was a great sin. Yurii Zhdanov wrote²⁸:

Lenin stated more than once that a recognition of the necessity of this or that phenomenon conceals in itself the danger of falling into objectivism. To a certain measure, I too did not avoid this danger.

Immediately after the session, about 3,000 scientists were dismissed from work. Russian genetics, which had produced splendid research recognized by the entire world, ceased to exist. Darkness fell on biological sciences in the Soviet Union. Nobody at that time could know how long that night would last — or whether he would survive until dawn. □

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- Soyfer, V. N. *Lysenko* to be published in Russian (Hermitage, Ann Arbor), and abridged in English (Rutgers University Press).
- Medvedev, Zh. *The Rise and Fall of T.D. Lysenko* (Columbia University Press, 1969).
- Joravsky, D. *The Lysenko Affair* (Harvard University Press, 1970).
- Roll-Hansen, N. *Science* **237**, 1329 (1985).
- Lysenko, T.D. *Bull. sort.-semenovod. Upravl.*, No. 4, 72–76; 77–80 (1923).
- Zaitsev, G.S. *Influence of Temperature on Cotton Development* (Proc. Turkestan Plant Breeding Station) Moscow-Leningrad. Promizdat (1927). (In Russian with English summary).
- Lysenko, T.D. *Trudy Azerbaidzhanskoi Tsentr opytno-selektsionnoi stantsii imeni tovarishsha Ordzhonikidze*, Part 3, Baku, 1–169 (1928).
- Lysenko, T.D. & Dolgushin, D.A. *Trudy Vsesoyuz. s'ezda po genetike, selektsii, semenovodstvu i plemennoumu zhivotnovodstvu* Vol. 3, 188–189 (Leningrad, 1929).
- Grigorev, V. *Pravda* No. 165 (4299), 21 July, p. 4, (1929).
- Shlikhter, A. *Pravda* No. 232 (4366), p. 3, (1929).
- Tulaikov, N.M. *Sel'skokhozyaistvennaya gazeta*, No. 212, p. 3 (12 November 1929).
- Popovskii, M.A. *Prostor, Alma-Ata*, No. 7 4–27; No. 8 98–118 (1966).
- Joravsky, D. *Slavic Rev.* **24**, (3), 381–394 (1965).
- Adams, M.B. *Dictionary of Scientific Biography*. Vol XV Suppl. 1, 505–513 (Scribner's, New York, 1978).
- Medvedev, A.A. *Novyi Mir*, No. 4, 226–234 (1967).
- Vavilov, N.I. *Smena*, Leningrad, 11 January (1929).
- Vavilov, N.I. *Sotsial. zemledelie*, No. 54, (616), p. 1 (24 February, 1931).
- Vavilov, N.I. No. 253 (815), (13 September, 1931).
- Vavilov, N.I. *Theoretical principles of the selection of plants*. Moscow-Leningrad vol. 1, p. 865 (1935).
- Konstantinov, P.N., Lysitsyn, P.I., Kostov, D. *Yarovizatsiya*, No. 5 (8), pp. 15–19 (1936).
- Vavilov, N.I., Speech of 17 June 1938 to the Presidium of the Lenin All-Union Academy of Agricultural Sciences. Archives of the LAAAS, document 450, p. 192, point 3.
- Vavilov, N.I., *Pravda*, No. 2 (6608), p. 2, 2 January (1936).
- Zhebrak, A.R., *Science*, 102, 2649 (1945).
- Vavilov, S.I. *Vestnik AN SSSR* No. 9, 26 (1948).
- Soyfer, V.N. *Kontinent* (Paris), No. 47, pp. 267–305, No. 48, pp. 263–297 (1986).
- Bazhanov, A.M. *On the Cultivation of Wheat, with a Description of the Crops grown in Russia*, Moscow. Izd. Moskovskogo Imperatorskogo Universiteta (1856).
- Kuperman, F.M., *Yarovizatsiya*, No. 2, pp. 101–105 (1940).
- Zhdanov, Yu. A. *Pravda*, No. 220 (10961), p. 5, 7 August (1948).

Where next is science heading?

The immediate future for science is bright, but the future may be clouded by poor public education and by too much competitiveness — the gist of a talk at Los Alamos on Tuesday.

Santa Fe

NATURALLY there is no way of telling where science is heading except in the most trivial sense. For example, one can safely guess that the new electron-positron accelerator (LEP) at CERN in Geneva, which will start operating a few weeks from now, will either discover the top quark, or it will not. In the first case, we shall all be a little more content. Otherwise we shall feel challenged and will start telling our governments that they should spend even more on high-energy physics than has been their custom for the past 40 years. Similarly, there are dozens of engineered biochemicals waiting in the wings for development to be complete and for the regulatory authorities to give their approval; if one of these should, for example, make good the defect we call schizophrenia, the world we live in would be different. So it will be when the pocket-sized mainframe computer is for sale.

The outstanding feature of those developments is that they lie within the reach of the development process as we know it. The top quark is simply an entity which, by evil chance, has not yet been discovered. To suppose that genetic engineering will yield a chemical that cures schizophrenia is an exaggeration born of wishful thinking; as things are, nobody knows whether there is an identifiable biochemical defect underlying the disease, when there are only the sketchiest notions of what it might be and when the aetiology of the disease is so poorly understood that nobody can be sure whether the defect is reversible. But the chance that these uncertainties might be resolved, the social importance of this and a host of other natural diseases is so great and the search for a better understanding of them is intellectually so interesting that the programmes of biomedical research now supported by our governments are self-evidently justified. Indeed, that is why some people argue that they are too small.

The interesting questions about the more distant direction of science cannot be as simply answered. Of course, the momentum of present developments will, to some extent, condition the fields in which discoveries are made. One obvious illustration is the present interest in the human genome. The immediate goal of producing a nucleotide sequence is justified by the potential benefits of being able simply to look up in a library genes and their control elements which are found, by

means of what will soon be straightforward biomedical research, to be involved in particular human diseases. But only a little reflection will show that long before the nucleotide map of the human genome is complete, human anthropologists will have recognized that these techniques provide them with a powerful new technique for understanding how the human population became what it is. More generally, it is only natural that the present concentration of talented people's energy in molecular biology will yield other benefits. We shall learn what is the genetic basis of speciation. We shall no doubt learn enough about the interactions of biomolecules to be able to simulate the origin of life. And, no doubt, molecular biology will soon move beyond its present preoccupation with the naming of the molecular parts of which organisms are made to a search for the dynamic relationships between them.

Similar prospectuses can easily be drawn up in other contexts. It is also clear that, despite the concentration of curiosity on the mechanism by which the outer skin of the Earth is kept habitable that has been growing since the International Geophysical Year, much more will now be learned not just about the stability of the biosphere, but about its manipulation. At least in principle, the discovery that anthropogenic emissions can affect the ozone layer works both ways: anthropogenic manipulation might be devised to protect it.

What the record of the past 40 years has shown is that the process of development has now become deliberate. It is possible to set goals and, within reason, to achieve them. That is what the construction of the first nuclear weapons showed. Since then, of course, we have also learnt, often painfully, that the achievement of a technical goal does not ensure that the products of development will be economically useful. Controlled thermonuclear fusion is an obvious case in which the technical feasibility of the process seems with difficulty assured, but the economics of the process are still an open question. Cold fusion, of course, is something else again.

But what lies beyond this predictable and mostly cheerful prospect for the next few decades? The simple answer is that there is no way of telling, given that the more distant future will hang on discoveries not yet made, but that presupposes that even the most important discoveries are accidental. But that is an over-simple

view. Take relativity for example.

It is in no way slighting of Einstein's reputation to assert that if he had not put forward the special theory of relativity in 1905, somebody else would have done so in a very short time. In that case, we even know who would have done so. H. A. Lorentz is one candidate, Poincaré so obviously another that there have been 80 years of vigorous argument on the question whether Poincaré anticipated Einstein. The obvious parallel, now, is the continuing search for a unified theory of fundamental forces. When, quite soon perhaps, there is a theory that really hangs together, it will seem a puzzle to us all that it took such a long time coming.

What that implies is that, even if it is not feasible to anticipate the process of discovery, it is at least possible to think of conditioning the scientific process to make it more fruitful of discovery. Since the Second World War, we have learned, or believe we have learned, a great deal in that direction. This is why we honour basic research — and why we are constantly engaged in the argument of the balance between basic research and its application.

Nobody can deny that the growth of science in the United States, and the greatest success that has flowed from it, stems from the commitment to basic research of successive administrations since the Second World War. It is remarkably enlightened that the Bush administration is seeking further increases of the budgets of agencies such as the National Science Foundation in a year when it should be bending all its energies on the reduction of the federal deficit. On the face of things, that augurs well.

But sadly, there is another side of the coin. Research prospers by the talents of those who enter the profession, but the state of public education and the social goals of young people threaten to restrict the supply of indigenous talent. Similarly, the competitiveness of the US research process, with its practical requirement that academic scientists should almost exclusively rely on competitive grants for their support, which explains the productivity of US science, has also had deleterious consequences. The flow of publications may have been increased, but not the exchange of ideas. There is in present circumstances a danger that science may no longer have room for scholarly people who do not care for the rat-race. That could be disastrous.

John Maddox

Cloning of the SR foot

William S. Agnew

FEW molecules associated with ion transport are more elaborate than the foot process of the sarcoplasmic reticulum (SR)¹⁻⁴, and few take part in more complex physiological events. In muscle fibres, this molecule serves as the ion channel responsible for the release of calcium stores in the SR to initiate contraction. The attention now focused on this channel, including the report of the cloning and expression of the rabbit skeletal muscle protein by Numa's group on page 439 of this issue⁵, promises to help explain how its activity is controlled by depolarization of the transverse tubule (T-tubule) membrane.

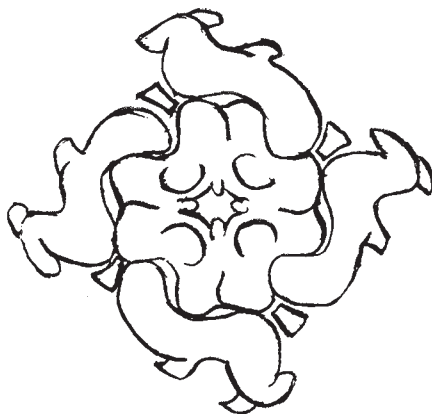
The foot structure is part of a molecular bridge which spans a gap of about 150 Å between the T-tubules and the terminal cisternae of the SR⁶. Large cytoplasmic extensions of the molecule evidently attach to the dihydropyridine (DHP) receptor complex in the T-tubule (see my recent News and Views article⁷). Biochemical isolation and reconstitution studies show the calcium-release channel to be identical to the receptor for ryanodine, a plant alkaloid that causes the mobilization of calcium in the SR. The receptor is enormous, being composed of four copies of a polypeptide of relative molecular mass >400,000 and has been well characterized by electron microscopy. Reconstructed three-dimensional images reveal a squarish molecule with large cytoplasmic extensions approximately 270 Å on each side, sometimes called a quatrefoil. This portion of the molecule, about 100 Å thick, has an intricate morphology with a distinct left-handed rotational asymmetry, giving a pinwheel appearance. Also present is a smaller base-plate, approximately 40 Å thick and 140 Å per side, which appears to represent the transmembrane portion. Reconstruction of the molecular interior suggests that a single central channel leads outwards to four internal vestibules through which calcium ions may pass. The molecular volume predicts an overall mass of 2.3×10^6 .

These morphological features are consistent with the new amino-acid sequence information deduced by Takeshima *et al.*⁵ in their report in this issue. The protein is encoded by a transcript of about 16 kilobases. The open reading frame is 15,111 bases, predicting a sequence of 5,037 amino acids and a computed molecular mass of 565,223 for the peptide and 2,260,892 for the tetramer.

Also consistent with the electron-microscope data, only the 500 or so amino acids at the carboxy terminus of the deduced sequence contain segments sufficiently hydrophobic to span the membrane. Takeshima *et al.* identify four potential

transmembrane sequences resembling the membrane-spanning segments of the nicotinic acetylcholine receptor. This supports the notion that there may be a single calcium channel formed by contributions from each of the peptide subunits. D. MacLennan and co-workers at the University of Toronto have also nearly completed sequencing not only the rabbit skeletal muscle protein, but also that from human skeletal muscle (personal communication). They conclude that there may be as many as ten transmembrane-spanning segments, perhaps more consistent with the fact that the channel is formed by four subunits rather than the five of the acetylcholine receptor.

Also in keeping with the molecular morphology is the observation that the amino-terminal 90 per cent of the peptide is hydrophilic, unlikely to enter the bilayer



Viewed from SR side of the membrane, the foot process exhibits a base-plate of about 140 Å on a side, 40 Å thick, lying on top of the quatrefoil which is 270 Å on a side and extends at least 100 Å into the cytoplasmic interior. This structure is formed of four copies of a 560,000 polypeptide (from ref. 1).

and certainly forming the enormous quatrefoil extension. Interestingly, just to the amino side of the hydrophobic segments, Takeshima *et al.* identify several possible consensus sequences for the binding of calcium, nucleotides and calmodulin, all of which have previously been implicated in modulating release of calcium.

There are various lines of evidence indicating that calcium release is triggered by the T-tubule DHP receptor⁷, perhaps much as envisioned by Chandler, Rakowski and Schneider⁸. In recent studies, Numa, Beam and collaborators⁹ provide strong evidence that this protein may act both as a slow (L-type) calcium channel and as the voltage-sensor for release. Because in skeletal muscle release does not depend on calcium influx, these appear to be separate functions of the same molecule⁷. The DHP receptor appears to be a stoichio-

metric complex of four or five peptide subunits, $\alpha 1$, $\alpha 2$, β , γ and possibly δ ^{7,10,11}. The $\alpha 1$ peptide resembles the large subunits of the voltage-sensitive sodium channel, probably folding into four membrane-spanning domains organized into a sort of 'false tetramer'¹⁰. Each membrane-spanning domain corresponds to an internally homologous segment of about 270 amino acids, and each carries a positively charged sequence (S4) which may act as the voltage-sensors. In sodium channels these four similar, but non-identical, segments are thought to specify the very intricate control of conductance¹². For excitation-contraction (EC) coupling, the picture will probably be significantly more complex.

To speculate on this complexity, one first recognizes the distinct possibility that each ryanodine receptor subunit might associate with a separate DHP receptor complex. If S4 regulation is direct, then rather than being controlled by four voltage-sensors, as are calcium and sodium channels, the ryanodine receptor could receive activating signals from as many as 16 voltage-sensors. This extreme possibility could make difficult the correlation of T-tubule charge movement and activation of release¹³. Furthermore, the activating signals must be communicated through an intramolecular distance of greater than 100 Å and may be subject to modulation.

Considerable evidence has accumulated concerning the role of diffusible small molecules in coupling. In this regard it is interesting that possible binding sites for calcium, ATP and calmodulin are localized between the cytoplasmic structure and the membrane-spanning domain. If confirmed by more direct experiments, this location is well-suited for modulation, perhaps weakening or strengthening conformational signals induced at the DHP receptor/ryanodine receptor interface. Donaldson and co-workers have obtained the intriguing result that calcium release is greatly sensitized to inositol (1,4,5) trisphosphate (InsP₃) when the T-tubule membranes of peeled muscle fibres are depolarized¹⁴. It is also possible that, under some circumstances, calcium-activated calcium release (cardiac muscle), or InsP₃ (smooth muscle) signals may be the primary release signal.

If the triadic assembly really is so complex, what are the prospects for ever being able to resolve the molecular mechanisms involved? One approach is to express all the cloned components, including the ryanodine receptor, in a cellular environment capable of supporting coupling, and then to examine functional changes resulting from mutations introduced by site-directed mutagenesis. Towards this end, Takeshima *et al.*⁵ have successfully expressed the cloned ryanodine receptor in Chinese hamster ovary cells in a form

MASS EXTINCTIONS

Amino acids and bolide impacts

John R. Cronin

capable of binding ryanodine. Biochemical studies soon to appear from Meissner's group suggest that ryanodine binding requires the assembled tetramer. Also, the dramatic results from replacing the genetically defective $\alpha 1$ peptide in mouse dysgenic muscle⁹ provides some cause for optimism that at some point the entire triadic complex will be reconstructed from the component cloned cDNAs.

Another possibility is to resort to more detailed structure determinations. The ryanodine receptor may offer the best opportunity so far for crystallographic studies of a complex ion channel. Only the smallest portion of the molecule lies in the membrane environment, where ordered three-dimensional lattices can be difficult to induce. The hydrophobic requirements of this region are likely to be easily satisfied by small amphiphiles, leaving an abundance of possible protein-protein interactions by the remaining 4,500 amino acids which may yield well-ordered crystals. Available pharmacological agents, including ryanodine, ruthenium red and even calcium might be used to lock the channel in opened or closed configurations, giving precise information as to the intramolecular rearrangements underlying gating. Not only would such information dramatically contribute to specific hypotheses about EC coupling, it would constitute the first direct information about gating of any ion channel.

There is one more tangential connection in which the new round of data should be considered: are there related mechanisms present in the central nervous system? It is certain that there are calcium-channel components in the central nervous system related to the T-tubule DHP receptor, with important roles in neurosecretion and perhaps postsynaptic events. Coupling plasma-membrane depolarizations directly to mobilization of internal calcium stores may be more general than currently thought, and molecules related to the ryanodine receptor should perhaps be actively searched for. □

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THE past few years have seen the publication of several very unexpected — in some cases, incredible — scientific reports: high-temperature superconductivity, antibody activity at extreme dilution and low-temperature fusion, to name but three. On page 463 of this issue¹, Zhao and Bada present results of amino-acid analyses of sediments from the Cretaceous/Tertiary boundary (marking one of the principal mass extinctions) that will be equally startling to organic geochemists and many planetary scientists. They find that Danish sediments spanning the narrow boundary layer contain two amino acids, α -aminoisobutyric acid and isovaline, that are relatively uncommon in biological materials but abundant in the organic-rich meteorites. They suggest that the body which collided with Earth 65 million years ago and left the tell-tale iridium residue may have been organic-rich, perhaps like a C-type asteroid or a comet. Such a possibility has interesting implications for the extinction and related atmospheric effects, and supports the idea that impact events could have supplied the Earth during a much earlier period with the raw materials for organic chemical evolution.

The data provided by Zhao and Bada are clear cut. They used standard chromatographic procedures to free the amino acids from inorganic salts and to do a preliminary fractionation of the apparently complex mix of amino acids in their extracts. (More comprehensive data are promised in a later paper.) The analyses were done using both high-pressure liquid chromatography and gas chromatography/mass spectroscopy and several checks were made beyond simple retention time comparisons to ensure the correct identification of α -aminoisobutyric acid (AIB) and isovaline. There seems little room for doubt that Zhao and Bada have correctly identified these amino acids, nor is there much reason to suspect sample contamination during analysis because, as far as we know, these amino acids are quite uncommon in the biosphere. Furthermore, Zhao and Bada used chiral analytic methods and found the isovaline to be racemic, containing both right- and left-handed forms, a result which is probably inconsistent with biological contamination. Thus they seem to have avoided the historic bugaboo of meteorite organic analyses — mistaking terrestrial contaminants for *bona fide* meteoritic constituents.

On the other hand, there are troubling points in the notion of meteorite delivery. The amounts of the amino acids found and their spatial distribution in the sediment samples are surprising in light of what might be expected if meteorite impact

were their source. (The survival of any amino acids, itself, is a surprise.) Zhao and Bada calculate that the amount of AIB found is about five times the maximum expected from a CM chondrite, an archetypal organic-rich meteorite, based on iridium content of samples. One would expect the survival of only a small fraction, at best, of the original molecular content of such an impactor, given the pyrolysis and combustion that must occur when a body of the necessary size slams into the Earth at nearly its cosmic velocity.

Zhao and Bada suggest that the impactor may have been much richer in organics (or poorer in iridium) than a CM chondrite, but such an effect must be enormous to allow for the expected impact destruction, to say nothing of later losses due to photochemical or biological decomposition while the amino acids were exposed at the Earth's surface. Amino acids are known to survive in fossilized material for perhaps millions of years, but in such cases they occur in shell or bone where they are protected by a tough, relatively impermeable inorganic coat². A meteorite or comet would seem to provide no comparable protection. The amino acids of CM chondrites are, by comparison, readily extracted into water without prior treatment to break down mineral phases.

According to Zhao and Bada, comet impact may provide a better explanation for their findings. Little is known, though, about the organic materials in comets; Kissel and Krueger³ state that the impact mass spectra obtained by the Vega mission to comet Halley give no evidence of amino acids. On the other hand, their elemental constituents are much more abundant in comets than in meteorites and occur there partially as the molecular precursors (aldehydes, HCN and NH₃) required for amino-acid synthesis by a Strecker synthesis. Clark⁴ has recently discussed the possibility of organic synthesis following comet infall.

A second anomaly in the data of Zhao and Bada is their finding that AIB and isovaline, although abundant in samples taken above the Cretaceous/Tertiary boundary, are completely absent in the boundary clay itself. Although the authors suggest that diffusion may explain this odd circumstance, the absence of amino acids from the boundary clay requires additional effects if the amino acids and iridium were originally deposited together.

Fortunately there are several experiments that would strengthen Zhao and Bada's hypothesis of an organic-rich impactor. First, amino-acid analyses of materials from other locales should be made to exclude the possibility of some

1. Wagenknecht, T. *et al.* *Nature* **338**, 167–170 (1989).
2. Lai, F.A., Erickson, H.P., E., Liu, O.Y. & Meissner, G. *Nature* **331**, 315–319 (1988).
3. Inui, M., Saito, A. & Fleischer, S. *J. biol. Chem.* **262**, 1740–1747 (1987).
4. Imagawa, T., Smith, J.S., Coronado, R. & Campbell, K.P. *J. biol. Chem.* **262**, 16636–16643 (1987).
5. Takeshima, H. *et al.* *Nature* **339**, 439–445 (1989).
6. Armstrong, C.F. *J. Cell Biol.* **47**, 488–499 (1970).
7. Agnew, W.S. *Nature* **334**, 299–300 (1988).
8. Chandler, W.K., Rakowski, R.F. & Schneider, M.F. *J. Physiol. Lond.* **254**, 285–316 (1976).
9. Tanabe, T., Beam, K.G., Powell, J.A. & Numa, S. *Nature* **336**, 134–139 (1988).
10. Tanabe, T. *et al.* *Nature* **328**, 313–318 (1987).
11. Ellis, S.B. *et al.* *Science* **241**, 1661–1664.
12. Stuhmer, W. *et al.* *Nature* (in the press).
13. Huang, C.L. *Physiol. Rev.* **68**, 1197–1247 (1988).
14. Donaldson, S.K., Goldberg, N.D., Walseth, T.T. & Hutteman, D.A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5749–5751 (1988).

fortuitous association in the Danish material. Second, meteoritic amino acids have been thoroughly investigated during the past 20 years and more than 50 have been identified which have no known terrestrial source⁵. The identification of some of these in Cretaceous/Tertiary-associated sediments would strengthen the argument for a meteoritic origin. Finally, isotope analyses have shown the amino acids of the Murchison meteorite, a CM chondrite, to be substantially enriched in ²H, ¹³C and ¹⁵N, relative to terrestrial organic matter⁶. Comparable enrichments might be expected if the boundary amino acids are, in fact, extraterrestrial. The results of experiments along these lines will be eagerly

awaited in follow-up to these intriguing observations by Zhao and Bada. Until this work has been extended, we shall all wait and wonder. □

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1. Zhao, M. & Bada, J.L. *Nature* **339**, 463–465 (1989).
2. Miller, G.H. & Hare, P.E. in *Biogeochemistry of Amino Acids* (eds Hare, P.E., Hoering, T.C. & King, K.) 415–443 (Wiley, Chichester, 1980).
3. Kissel, J. & Krueger, F.R. *Nature* **326**, 755–760 (1987).
4. Clark, B.C. *Origins of Life* **18**, 209–238 (1988).
5. Cronin, J.R., Pizzarello, S. & Cruikshank, D.P. in *Meteorites and the Early Solar System* (eds Kerridge, J.F. & Matthews, M.S.) (University of Arizona Press, 1988).
6. Epstein, S., Krishnamurthy, R.V., Cronin, J.R., Pizzarello, S. & Yuen, G.U. *Nature* **326**, 477–479 (1987).

PROTEIN STRUCTURE

Ions, gates and channels

N. Michael Green

If the three-dimensional structure of a protein is not known, the information that can be derived from site-directed mutants is often disappointing. Even when the structure is known, the effects of alteration can be difficult to interpret, particularly if mutants are characterized by only a single functional assay. A new study from the laboratories of MacLennan and of Inesi on the effects of mutating charged and polar residues in the transmembrane segments of the Ca²⁺-transporting ATPase of sarcoplasmic reticulum, reported on page 476 of this issue¹, is more than usually rewarding. This ATPase belongs to the family of P-type ion pumps, about 25 of which have now been sequenced. Our model of the Ca²⁺ pump², based on amino-acid sequence, affinity labelling of functional sites, fluorescence energy transfer and electron micrographs, has led to

speculations about function. These speculations can now be made more precise.

The cytoplasmic region of this ATPase is suggested to have a kinase-like domain structure, in which movements of domains following phosphorylation are transmitted to a Ca²⁺-binding gate, from which the gated ion can escape only to the exterior. Indirect evidence has led to the suggestion that Ca²⁺ is bound in the amino-terminal quarter of the protein, but no precise location for the binding site could be assigned. Charged residues present in the hydrophobic segments provide a potential polar channel, but it was not clear whether the gate is integral to the channel or whether it is in the cytoplasmic region, closer to the phosphorylation site. The presence of many negative charges in the α -helical stalk segments, which connect the cytoplasmic domains to the membrane, has led to speculation that the gate is located there². Experiments to test this idea³ show that replacement of about 20 negative charges in the stalk, either singly or in groups, does not affect Ca²⁺ uptake by microsomes from transfected cells (Fig. 1).

The gate must therefore be located somewhere else. The obvious place to look was in the charged and polar groups in transmembrane helices. In their paper in this issue¹, Clarke *et al.* report that only six residues, three glutamic acids, an aspartic acid, an asparagine and a threonine, which are located in helices M₁, M₃, M₆ and M₈, are crucial. Minimal changes in any of these block both transport and calcium-activated phosphorylation by ATP. In the reverse direction, phosphorylation by inorganic phosphate remains at the normal level and is not blocked by Ca²⁺. Ca²⁺ bound to the high-affinity sites usually prevents the back reaction by stabilizing the E_i conformation.

These results can all be explained if the six crucial polar groups contribute to the

binding sites for the two transported calcium ions. Although indirect effects cannot be excluded, the observation that assays for four different functions are similarly affected can be most simply explained if changing any one of the six residues disrupts the site. Because the groups are all in the middle segment of the helices, this gives strong support to a gate formed from binding sites which are an integral part of the channel, a feature common to many models⁴. The many available oxygen ligands suggest that peptide carbonyls and water molecules are not extensively involved, and does not support models for an aqueous channel such as those put forward by Edmonds⁵. As well as these six polar groups, there are six positive and five negative charges nearer to the membrane surfaces which can be changed without affecting ion transport. This shows that an exchange system of ion pairs⁶ is not important in controlling the passage of Ca²⁺, although it could modulate access to the binding sites.

Formation of a channel which would include M₁, M₃, M₆ and M₈ places limitations on the organization of the transmembrane helices. If we assume a double layer of helices, by analogy with bacterial rhodopsin and consistent with the asymmetrical distribution of protein deduced from electron microscopy of crystalline arrays of the calcium pump⁷, two types of array can be distinguished—cyclic (Fig. 2a) or alternating (Fig. 2b). Many versions of each type, as well as

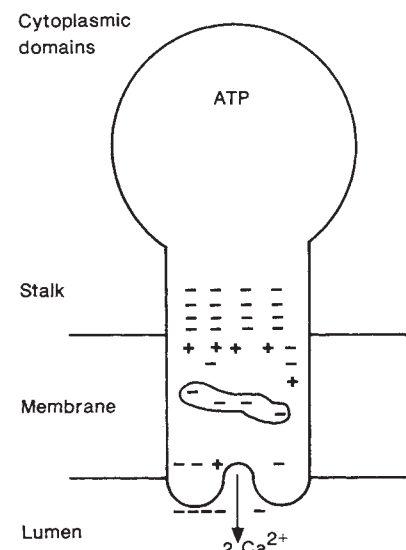


FIG. 1 Approximate locations of charged groups in the stalk and transmembrane region which have been mutated. Only the enclosed cluster is crucial for calcium transport.

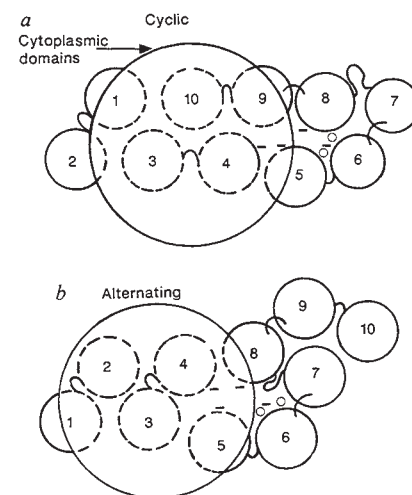


FIG. 2 Two examples from the many possible arrangements of transmembrane helices. Critical charges (–) and oxygen ligands (O) of the gating region are indicated. a, The two-layered cyclic arrangement resembles that in bacterial rhodopsin. b, An alternating arrangement places helices 2, 3, 4 and 5, which connect the cytoplasmic domains, in a more compact cluster. Connections 1–2, 3–4, 5–6, 8–9 and 9–10 are appropriately short and the more hydrophobic helices (1, 2, 7 and 9) are peripheral. The total number of transmembrane segments is still under discussion⁸. The ten shown here represent a maximum.

three-layered structures, are possible, so Fig. 2 focuses attention on only a few. Electron microscopy of frozen-hydrated crystals may eliminate some of these.

The great merit of the experiments of Clarke *et al.*¹ is that not only do they provide strong evidence for a gate which is integral with the channel, but they also point the way to further progress. One of the problems with site-directed mutagenesis is the choice of residues that might generate interesting mutants. The success rate should now be greatly increased by using the critical sites identified by Clarke *et al.* as starting points for exploring the surfaces of helices that might line the channel. The results so far have been obtained with only a few micrograms of each mutant. Purification of larger amounts would allow our extensive knowledge of the kinetics of the transport process to be more fully exploited.

The results are relevant to other P-type ion pumps. Although these show 30–50 per cent sequence identity in the cytoplasmic region⁸, their transmembrane segments have little more than their hydrophobicity in common. Only the Na⁺/K⁺ and H⁺/K⁺ pumps can be unequivocally aligned. It is therefore particularly striking that the general pattern of the critical polar groups identified by Clarke *et al.*¹ persists in all but the bacterial pumps. The variety of ion-transport processes that can be coupled to a homologous ATP-driven phosphorylation mechanism is an intriguing feature of these pumps. The new observations bring this puzzle into sharper focus. It may be resolvable by analogous studies of mutants of other members of the family⁹. □

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1. Clarke, D.M., Loo, T.W., Inesi, G. & MacLennan, D.H. *Nature* **339**, 476–478 (1989).
2. Brandl, C.J., Green, N.M., Korczak, B. & MacLennan, D.H. *Cell* **44**, 597–607 (1986).
3. Clarke, D.M. *et al.* *J. biol. Chem.* (in the press).
4. Tanford, C. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2882–2884 (1982).
5. Edmonds, D.T. in *Biological Membranes* Vol. 5 (ed. Chapman, D.) 350–387 (Academic, London, 1984).
6. Green, N.M., Taylor, W.R., Brandl, C.J., Korczak, B. & MacLennan, D.H. in *Calcium and the Cell* Ciba Fdn Symp. **122**, 93–113 (1986).
7. Taylor, K.A., Dux, L. & Martonosi, A. *J. molec. Biol.* **187**, 417–427 (1986).
8. Serrano, R. *Biochim. biophys. Acta* **947**, 1–28 (1988).
9. Portillo, F. & Serrano, R. *EMBO J.* **7**, 1793–1798 (1988).

- FOR 25 years, researchers have tried without much success to identify the cell surface receptors that determine the binding and entry into cells of C-type leukaemia and sarcoma viruses. In the 19 May issue of *Cell*¹, Lorraine Albritton and colleagues in Jim Cunningham's laboratory at Harvard Medical School describe the cloning and characterization of the receptor for the murine ecotropic leukaemia virus (MuLV-E). The only other retrovirus receptor identified to date is the CD4 antigen recognized by human and simian immunodeficiency viruses^{2–6}.

VIROLOGY

Leukaemia virus receptor

Robin A. Weiss

MuLV genomes of laboratory mice are transmitted through the germ line as proviruses integrated in the chromosomal DNA. After activation to infectious form, some strains of MuLV infect mouse cells whereas others can only infect cells of foreign species. Jay Levy⁷ coined the terms 'ecotropic' (MuLV-E) and 'xenotropic' (MuLV-X) to denote this difference in viral host range. Some wild mice also harbour 'amphotropic' virus (MuLV-A) that infects both murine and foreign cells. Susceptibility to infection is determined by the presence of cell surface receptors specific to the gp70 envelope antigens of MuLV-E, MuLV-X and MuLV-A, respectively. Albritton *et al.*¹ used this fact to clone the mouse receptor gene by transfecting murine DNA into the

human EJ bladder carcinoma cell line, together with the *gpt* gene, thus allowing the selection of cells in medium containing mycophenolic acid. The surviving cells were then exposed to a recombinant mouse sarcoma virus (MSV-NEO) genome carrying the G418 drug-resistance marker in an ecotropic envelope. Only those human cells transfected with and expressing the ecotropic receptor gene should be infectible with the MSV-NEO genome and thus become G418-resistant.

Of about 20,000 *gpt*-resistant human cell clones, Albritton *et al.* recovered 12 G418 resistant colonies, only one of which was susceptible to secondary infection with another ecotropic vector carrying methotrexate resistance. They derived secondary transfectant EJ cells from this susceptible clone, eventually cloning the receptor gene from one of these secondary transfectants using highly repetitive murine DNA sequences as tags. Complementary DNA libraries were then used to identify a complete receptor gene transcript, and transfection of the cDNA clone also conferred susceptibility to MuLV-E on human cells. The cDNA for the receptor contains an open reading frame which encodes a protein of 622 amino acids and of relative molecular mass 67,000. The putative protein is extremely hydrophobic containing up to 14 membrane-spanning domains. This

protein is not related to any previously deposited in gene and protein sequence data banks.

This study opens the way for a detailed analysis of the MuLV-E receptor and investigation as to whether receptors for other C-type retroviruses belong to the same family. The normal function of this newly discovered membrane protein will also be of interest. With its multiple hydrophobic domains resembling transmembrane sequences, it may act as a transporter molecule⁸.

Retrovirus receptor genes were first defined in chickens using classical mendelian segregation of virus susceptibility^{9–11}. Later, somatic-cell hybrids between susceptible and resistant species were used to locate the MuLV-E receptor gene to murine chromosome 5 (refs 12, 13). It is satisfying that the newly cloned gene resides on this chromosome¹. Somatic hybrids have also been used to locate the receptor gene for human T-cell leukaemia viruses types I and II to human chromosome 17 (ref. 14), and several groups are now trying to identify this receptor through expression cloning systems.

It is interesting that the recently cloned receptors for poliovirus¹⁵ and rhinovirus^{16,17} (ICAM-1) belong to the immunoglobulin superfamily, like the HIV receptor (CD4). The Epstein-Barr virus receptor is the C3D complement receptor¹⁸, another cell surface molecule with immunological functions. The MuLV-E receptor clearly is an entirely different kind of molecule. Some retroviruses may use more than one receptor. McGrath and Weissman¹⁹ postulated that thymotropic MuLV strains may recognize T-cell receptors, and we recently demonstrated CD4-independent infection of glial and muscle cells by HIV²⁰. Given the great diversity of retroviruses with respect to host range and cellular tropism, there may be new kinds of receptor awaiting discovery. □

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- Albritton, L.M., Tseng, L., Scadden, D. & Cunningham, J.M. *Cell* **57**, 659–666 (1989).
- Dalglish, A.G. *et al.* *Nature* **312**, 763–767 (1984).
- Klatzmann, D. *et al.* *Nature* **312**, 767–768 (1984).
- McDougal, J.S. *et al.* *Science* **231**, 382–385 (1986).
- Maddon, P.J. *et al.* *Cell* **47**, 333–348 (1986).
- Sattentau, Q.J. *et al.* *AIDS* **2**, 101–105 (1988).
- Levy, J.A. *Science* **182**, 1151–1153 (1973).
- Lodish, H.F. *Trends biochem. Sci.* **13**, 332–334 (1988).
- Payne, L.N. & Biggs, P.M. *Virology* **24**, 610–616 (1964).
- Rubin, H. *Virology* **26**, 270–276 (1965).
- Vogt, P.K. & Ishizaki, R. *Virology* **26**, 664–672 (1965).
- Ruddle, N.H. *et al.* *J. exp. Med.* **148**, 451–465 (1978).
- Oie, H.K. *et al.* *Nature* **274**, 60–62 (1978).
- Sommerfelt, M.A. *et al.* *Science* **242**, 1557–1559 (1988).
- Mendelsohn, C.L., Wimmer, E. & Racaniello, V.R. *Cell* **56**, 855–865 (1989).
- Greve, J.M. *et al.* *Cell* **56**, 839–847 (1989).
- Staunton, D.E. *et al.* *Cell* **56**, 849–853 (1989).
- Fingerroth, J.D. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 4510–4514 (1984).
- McGrath, M.S. & Weissman, I.L. *Cell* **17**, 65–75 (1979).
- Clapham, P.R. *et al.* *Nature* **337**, 368–370 (1989).

Probing biological structure

R.A. Crowther

THE scanning tunnelling microscope, for which Binnig and Rohrer received a share of the 1986 Nobel prize for physics, has produced spectacular images of the atomic details of surface structure on metals and semiconductors. A recent study¹, for example, reveals the patterns of adsorption of iodine atoms on single crystals of platinum imaged in air. Current reports of the application of scanning tunnelling microscopy to imaging DNA by Arscott *et al.* on page 484 of this issue², and of its cousin the atomic force microscope to imaging the polymerization of fibrin by Drake *et al.* in a recent issue of *Science*³, suggest that such scanning probe microscopes will also provide important information about biological structures and their assembly.

Compared with conventional electron microscopy, in which the electron beam produces severe radiation damage in biological specimens, scanning probe microscopes are much less damaging. Unlike diffraction techniques, which reveal the average properties of fairly large volumes of the sample, scanning probe microscopy allows imaging of individual atomic sites and thus provides details of local structure. Most important, the probes can work when the specimen is in water, a crucial advantage in the study of biological assemblies.

The scanning tunnelling microscope (see ref. 4 for a review) uses an atomically sharp metal probe to scan over the surface of the specimen with a gap of a few angstroms, so that a tunnelling current flows when a bias voltage is applied

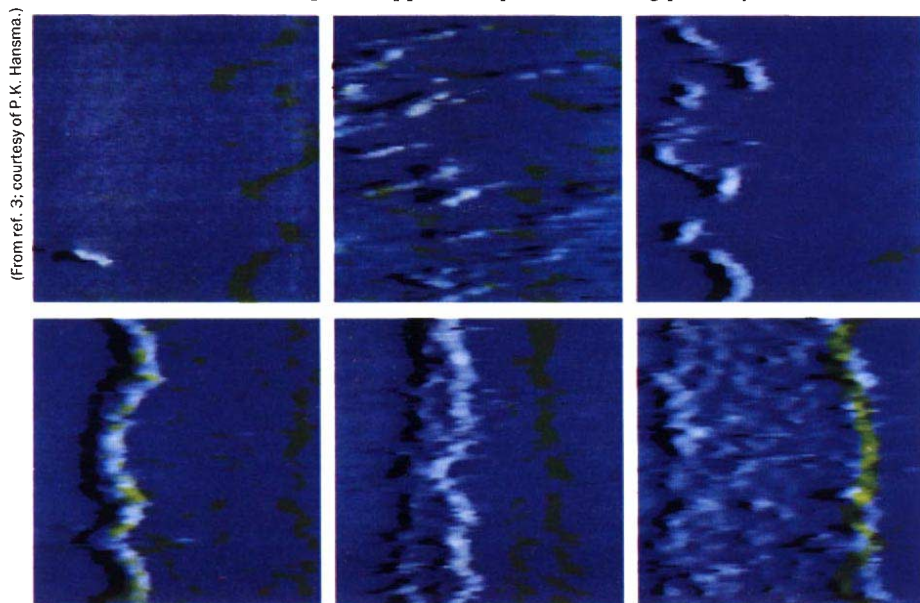
between tip and specimen. The specimen is scanned in a raster by applying a varying voltage to the piezoelectrically activated crystal on which the probe is mounted; in constant-current mode the tunnelling current is used in a feedback loop to keep the probe at constant height above the specimen during the scan. The tunnelling current is such a rapidly varying function of the gap size, that the image produced from the probe height signal is essentially a topographical map of the specimen, accurate to a fraction of an angstrom, although it also depends on the work-function. For the tunnelling current to flow, the specimen needs to be an electrical conductor and this had previously been achieved by covering the biological specimen, such as the recA-DNA complex⁵, with a thin metallic coat. But this procedure negates one of the principal advantages of the approach, which is its ability to examine native biological material. Recently⁶, the recA-DNA complex has been imaged without metal coating after deposition on a conducting substrate, although the mechanism by which the tunnelling current flows is not understood. Images of coated and uncoated recA-DNA complexes are similar, with a level of detail corresponding to the helical packing of the recA protein subunits apparent in both cases⁶.

Images in the scanning tunnelling microscope^{2,7} of various kinds of naked DNA and RNA show dimensions in reasonably good agreement with X-ray diffraction measurements, despite the specimens being partially air-dried. More

important, Fourier transforms of the images show regular spacings out to 7 Å, indicating that at least some features of the repeating structure are preserved to this degree in unstained, unshadowed images of groups of a few molecules. Furthermore, it is possible to detect deviations from the regular helical structure, giving hope that local features of the structure can be seen, though caution is needed here until the variations can be shown to be reproducible and sequence specific. The scanning tunnelling microscope image of a portion of Z* DNA fits better by model building² with an electrostatic potential surface than with a van der Waals surface of the molecule, which should give some clues about the detailed nature of the image. Studies of liquid crystals⁸ suggest that image contrast is produced by modulation of the local work function of the substrate by the polarizable molecular adsorbate.

The atomic force microscope also probes surface structure, but in a more direct way. By mounting a minute shard of diamond weighing a few nanograms on a microfabricated cantilever arm, the surface details of the specimen can be read out in much the same way as a gramophone record is played with a stylus. The deflections of the probe as the surface is scanned are so small that they originally had to be detected by a scanning tunnelling microscope⁹. Such a piggy-back arrangement is difficult to operate routinely and a more satisfactory atomic force microscope has now been developed³, which uses an optical level system to monitor directly the small displacements of the cantilever. With a mechanical probe, the specimen no longer needs to be conducting and the tracking force is so weak that it seems that the surface can be scanned repeatedly without damaging it. Covering the specimen with water is a positive advantage, as it reduces the adhesive force between probe and surface arising from contaminants when the surface is scanned in air.

The scene was thus set for investigation of a biological specimen and Drake *et al.*³ recorded a series of atomic force microscope images in real time of the polymerization of the blood clotting protein fibrin (see figure). The resolution achieved is nothing like the 5-Å spacings they show in imaging the surface of mica, but nevertheless the formation of chains of monomers



Images in the atomic force microscope showing clotting of the human blood protein fibrinogen in real time. The sequence starts, top left, before introduction of the clotting protein thrombin and runs through to, bottom right, 33 min after introduction. Each image 4,500 × 4,500 Å.

1. Schardt, B.C., Yau, S.-L. & Rinaldi, F. *Science* **243**, 1050-1053 (1989).
2. Arscott, P.G., Lee, G., Bloomfield, V.A. & Evans, D.F. *Nature* **339**, 484-486 (1989).
3. Drake, B. *et al.* *Science* **243**, 1586-1589 (1989).
4. Hansma, P.K., Elings, V.B., Marti, O. & Bracker, C.E. *Science* **242**, 209-216 (1988).
5. Amrein, M., Stasiak, A., Gross, H., Stoll, G. & Travaglini, G. *Science* **240**, 514-516 (1988).
6. Amrein, M., Durr, R., Stasiak, A., Gross, H. & Travaglini, G. *Science* **243**, 1708-1711 (1989).
7. Lee, G., Arscott, P.G., Bloomfield, V.A. & Evans, D.F. *Science* **244**, 475-477 (1989).
8. Spong, J.K. *et al.* *Nature* **338**, 137-139 (1989).
9. Binnig, G., Quate, C.F. & Gerber, C. *Phys. Rev. Lett.* **56**, 930-933 (1986).

(From ref. 3; courtesy of P.K. Hansma.)

and the aggregation of the chains into a mini-clot are clearly seen. To visualize such an assembly process at this level of detail would be extremely difficult, if not impossible, with any other available technique. The main limitation seems to be movement of the protein molecules and the difficulty of getting them to bind onto the mica support. The tracking force keeping the probe on the surface may cause the molecules to move during scanning, but the authors claim that with further miniaturization of the probe, the tracking force can be reduced still more. There is obviously wide scope for develop-

ment of specimen-preparation techniques appropriate to the new microscopes. Although limited to producing topographical images, it seems that scanning probe microscopy will become an important tool for investigation of native biological structures and of their assembly, in those situations where the growing assembly can be made to adhere firmly to a flat surface without damage to the specimen. □

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OCEAN DRILLING PROGRAM

Plumbing the Pacific sinks

Leg 125 shipboard scientific party*

SEAMOUNTS, it is generally thought, are the result of volcanism at the sea floor. But on Leg 125, we drilled some remarkable and quite different seamounts between the deep ocean trenches and active island-arc volcanoes of the Mariana and Izu-Bonin regions south of Japan (see map). These seamounts are draped not by lavas but by muds derived from mantle peridotite and protrude from the sea floor because of their low density. The muds entrain blocks of peridotite and volcanic rock from the underlying crust and mantle, and fluids with a high concentration of hydrocarbons that also have a deep-seated origin.

The areas of study, known as forearc terranes, overlie the shallowest parts of the subduction zone along which Pacific oceanic lithosphere bends downwards and descends deep into the mantle. The mantle in the wedge-shaped area above this subducted lithosphere has partially reacted with water to form hydrated magnesium silicate — serpentine — and the resulting low density material has risen to form a zone of seamounts between 50 and 150 km from the trench. We drilled on two of these seamounts, one in the Mariana (sites 778–780) and one in the Izu-Bonin (sites 783 and 784) region (see map). Previous surveys had suggested that the Mariana seamount resembled a volcano with flows of serpentine draping its surface and fluids escaping from its summit. Drilling into the flanks of the seamount revealed spectacular plastic deformation fabrics within the serpentine that confirmed that it had flowed.

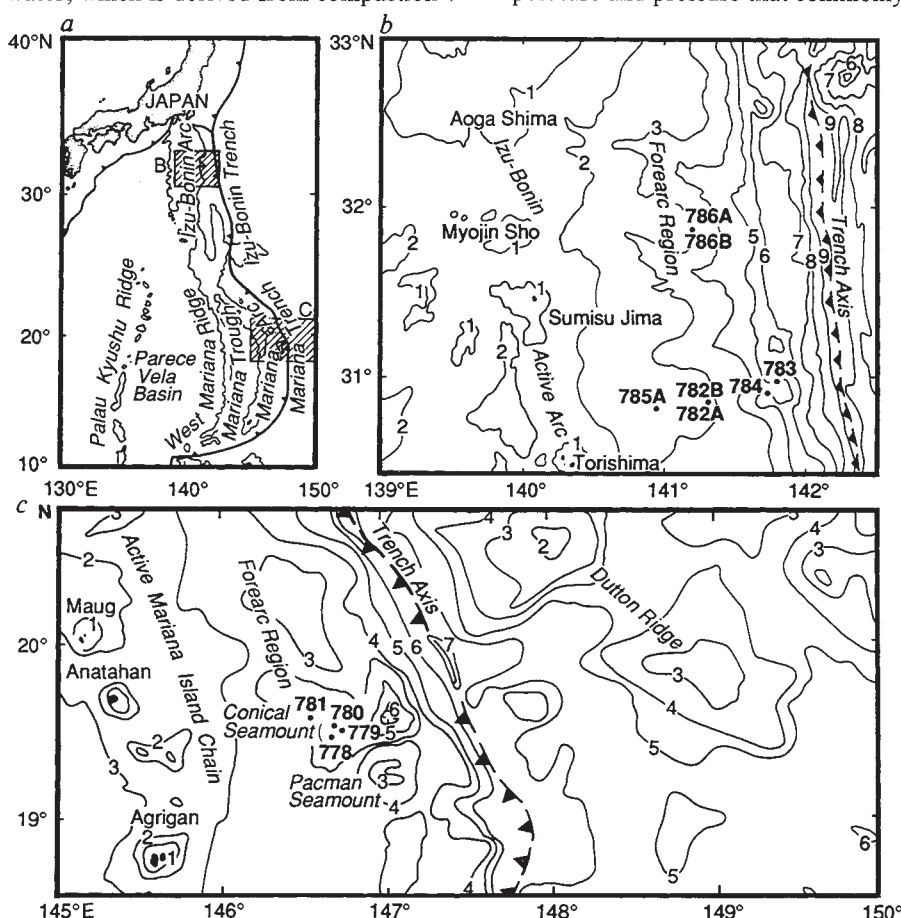
We recovered from the summit soft serpentine mud containing solid, variably hydrated, blocks of mantle peridotite and fragments of volcanic rock. Experiments performed on board showed this mud to have sufficient buoyancy and strength to both support the entrained blocks and rise to the sea floor. This material then flows down the side of the edifice because of its low slope stability. Microfossils entrained

in one of the flows on the Mariana seamount date as Pleistocene (about 1.6 million years ago (Myr)) suggesting that the seamount has been 'active' for some time. By contrast, the Izu-Bonin seamount is at least 10 Myr old and is probably 'extinct'.

The water that converts mantle rock to serpentine may originate as sea water, which penetrates the forearc crust and mantle from above, or as subducted water, which is derived from compaction

and dehydration of subducted oceanic crust and sediment and which penetrates the forearc mantle from below. Pore waters recovered from the serpentine-rich muds in the Mariana seamount at depths up to 314 m below the sea floor show that both sources are represented and that the waters are flowing slowly up through the seamount and also seeping into the ocean at its summit. The pore waters have less than half the chloride and bromide of seawater, pH values up to 12.6 (as opposed to 8 in sea water) and high concentrations of carbonate, bicarbonate, sulphate ions and the light hydrocarbons, methane, ethane and propane. Ethane and propane are particularly significant: they form only at high temperature, probably above 100 °C, and therefore probably originated at depth, either in the subducted plate or during upward flow of fluids through the overlying mantle.

Geochemical fingerprinting of the volcanic fragments suspended in the serpentine shows that few of these fragments are genetically related to the mantle in which they were entrained; nor do many have the composition of the forearc crust sampled at sites 784 and 786. Most have the composition of basalts from normal oceanic crust and were metamorphosed under conditions of moderate-to-low temperature and pressure that commonly



Location of Leg 125 sites: a, the regional setting of the Izu-Bonin and Mariana forearcs; b, the precise setting of the Izu-Bonin drill sites, sites 782–786; and c, the precise setting of the Mariana drill sites, sites 778–781. (Contours in kilometres depth.)

prevail where cold oceanic lithosphere is subducted. Some of the volcanic fragments may therefore represent pieces of Pacific crust that have been accreted onto the forearc and brought to the surface by the serpentinite bodies.

Despite the metamorphic evidence, however, the concept of a cold forearc is still open to question. Drilling at site 781, on the lowermost flank of the Mariana serpentine seamount, intersected a basalt lava of late Pliocene or younger age (less than 3 Myr). This is the youngest forearc volcanic rock yet reported and of similar age to some of the cold serpentine flows extruded from the summit of the same seamount. This paradox is still not resolved: either the forearc has a highly variable and complex thermal structure or the basalt formed from a lateral injection of magma from the volcanic arc nearly 100 km to the west.

Drilling the sediments and volcanic basement in the central part of the Izu-Bonin forearc has enabled us to study the crust that may have overlain the mantle exposed in the serpentine seamounts. At site 782, a thick sedimentary sequence was recovered containing over 100 layers of volcanic ash which document how the intensity of volcanic activity of the region has varied with time. Preliminary results suggest that there were two periods of greatest intensity, one from the late Miocene to the present day (5–0 Myr) and one during the Eocene–Oligocene (about 45–30 Myr). At site 786, an 828-m hole was drilled through a thin sedimentary

cover into the depths of an old submarine volcano. The rocks recovered include a wide range of lava types (ash flows, massive flows and pillow lavas) intruded by dykes and sills that represent the feeder channels to these lavas. In the deepest part of the hole, dykes and sills dominate lavas, and the abundant sulphide-filled fractures indicate that the volcanism was probably accompanied by intense hydrothermal activity. Microfossil dating of sediments intercalated within the lavas give a middle Eocene age (42 Myr), demonstrating that this volcanic event took place shortly after subduction started. The unusual, magnesium-rich (boninitic) composition of many of the lavas and dykes is also thought to be a characteristic of magma genesis in the earliest stages of subduction. It is thought that the mantle drilled at the seamount sites probably represents the residue from the melting event that formed this type of Eocene forearc crust. □

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OXIDE SUPERCONDUCTORS

Occam's razor and holy grails

Philip B. Allen

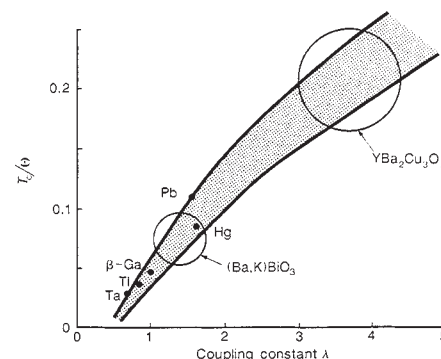
SOLID-STATE physicists have long regarded the search for superconductors with a higher transition temperature (T_c) as a holy grail. After 13 years with $T_c \leq 23$ K, the new CuO-based layer compounds with $T_c \leq 125$ K came as a stunning surprise, and continue to challenge both theory and experiment to unravel the mechanism. The resulting renewed attention to a similar family of superconductors based on cubic BaBiO₃ has been very fruitful¹, yielding T_c up to 30 K by partial substitution of barium by potassium. Evidence is accumulating that nature flouts Occam's razor most outrageously: oxide superconductors based on BaBiO₃ may be completely different from those based on CuO. Specifically, BaBiO₃ seems to be a virtuoso conventional (electron–phonon) superconductor, an interpretation supported by new tunnelling² and neutron scattering^{3,4} data from Hinks and co-workers, whereas YBa₂Cu₃O₇ and related metals are more probably novice players in a new and still mysterious consortium.

Conventional superconductors can usefully be plotted on the graph shown; the precise coordinates are best measured by the technique of 'quasiparticle tunnelling spectroscopy'. Unfortunately, fabrication of good tunnel junctions is an art slow to mature, and we do not yet have interpretable data for any new superconductor. The vertical axis is T_c normalized to the temperature scale $\Theta = \hbar \langle \omega^2 \rangle^{1/2} / k_B$ set by the lattice vibrations of frequency ω , and the horizontal scale is the coupling constant λ ; k_B is Boltzmann's, and $\hbar$ Planck's, constant. Representative data for various elements are shown, and theoretical calculations have given *post facto* explanations for the measured parameters. Values of T_c in simple cases can now be predicted *ab initio* but with a fairly large uncertainty.

It is often remarked that 25–30 K is an upper limit to T_c by a conventional mechanism, and years of frustrated effort have given this empirical validity. In terms of the coordinates on the graph, one can rationalize a limit to T_c based on the fact that λ

and Θ are partly coupled. As λ increases, Θ tends to decrease, restricting T_c , or worse, causing a lattice instability or a first-order phase change. These phenomena are too complicated to be captured in a simple mathematical relation. Theory provides no absolute limit to T_c , but experience has suggested guidelines. For $T_c \approx 28$ –30 K in Ba_{1-x}K_xBiO₃, a circle on the graph denotes reasonable guesses for conventional coordinates. The required value of λ , 1.0–1.5, is high but no larger than seen in 'strong-coupling' systems such as lead and mercury. The extraordinarily high T_c derives from an unusual strong coupling between electrons at the Fermi level (the maximum energy of occupied electron states) and oxygen vibrations.

There are several reasons why this picture is reasonable. First, reducing x to zero gives the semiconducting parent compound BaBiO₃. This material would be metallic according to band theory⁵, having a half-filled conduction band allowing charge to flow, if it occurred in an undistorted cubic structure. But a strong electron–phonon interaction (involving Bi–O bond-stretching distortions) causes⁶ a textbook example of a Peierls distortion of the lattice^{3,4}, doubling the unit cell, splitting the conduction band in two which inhibits charge flow, and making a semiconductor. As x increases, the Peierls distortion weakens and goes away, leaving a metal with a strong interaction between the highest occupied electron states and oxygen vibrational modes — a perfect candidate for high T_c . Second, this picture has been fleshed out with detailed calculations⁶ of λ and $\langle \omega^2 \rangle$ which confirm the credibility of $\lambda \geq 1.0$. Third, recent data are consistent with this interpretation: optical data by Sato *et al.*⁷ suggest conventional metallic behaviour in the superconducting part of the phase diagram, and tunnelling spectra by Zasadzinski *et al.*² suggest



Normalized transition temperature T_c/Θ versus coupling constant for several superconductors. The shaded region shows a range of permitted values obtained by varying the hidden coupling constants — the coulomb repulsion μ^* (in the range 0.10–0.15) and the shape of $\alpha^2 F(\omega)$, the electron–phonon spectral function. Two shapes were used: that of ref. 5 and that of $g(\omega)$, the phonon density of states, measured by W. Reichardt and W. Weber (ref. 4).

ARCHAEOLOGY

Getting into the groove

Paul G. Bahn

a conventional energy gap and strong coupling between electrons and oxygen vibrations.

Why can such a picture not be extended to CuO systems? In fact, such a picture was presented for La_2CuO_4 by Weber⁸, but subsequent work has undermined the premises of this work severely. The semiconducting nature of pure La_2CuO_4 is now known not to be a Peierls effect but a magnetic effect. The most recent band theories⁹ give remarkable agreement with experiment for vibrational frequencies and find only moderate coupling of Fermi-surface electrons to Cu–O stretching modes. In addition, a powerful set of arguments makes the electron–phonon interaction an unlikely candidate. First, T_c is too high. The conventional coordinates for $\text{YBa}_2\text{Cu}_3\text{O}_7$ ($T_c = 92$ K) are shown as the large circle on the graph. A value $\lambda \approx 3$ –4 is needed which, although not forbidden, is unheard of and would probably drive a lattice instability. Second, electrical resistivity of the normal material resembles the behaviour of propagating charge carriers with long mean free paths (≥ 30 Å at room temperature). This is hard to reconcile¹⁰ with strong coupling to the lattice such as $\lambda \approx 3$ –4.

Third, isotopic substitution of oxygen, changing the phonon frequencies, seems to have a small effect in CuO-based systems and a larger one in BaBiO_3 . Fourth, and most interesting, is the close correlation between superconducting compounds and related materials with strong antiferromagnetic ordering on Cu^{2+} ions. Long-lived magnetic moments on copper atoms would weaken or destroy ordinary superconductivity. Many unconventional models have been proposed in which antiferromagnetic coupling creates superconductivity. These theories have not yet matured and do not yet bear closely on experiments. Many other unconventional mechanisms have been proposed, but are similarly undeveloped.

If it is indeed true that different mechanisms are needed for different oxides, then nature has played a very clever trick: we were looking for a holy grail and now look foolish holding two. □

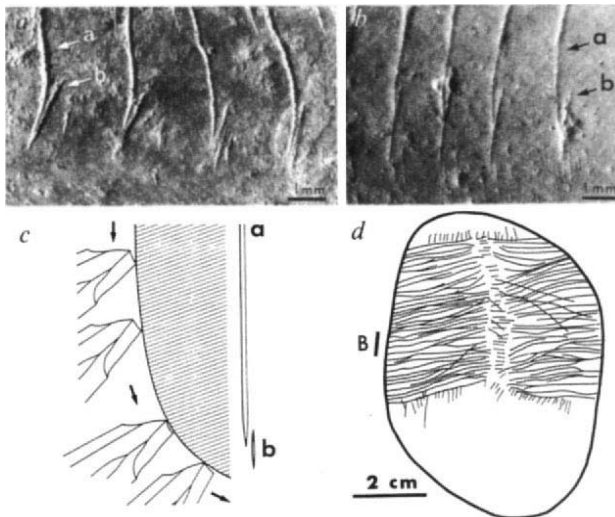
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A DEBATE between two researchers^{1–3} should lead to a clearer picture of whether and how we can recognize engraved notations or calendars on prehistoric artefacts, by establishing the evidence that reveals if series of marks were accumulated slowly or made rapidly in one session. The latest research on such engraved marks also provides a great deal of other information about how they were made.

'Experimental' engravings, resin replicas of prehistoric engraved pebbles and scanning electron microscope studies are providing clear criteria for the identification of marks made by different tools, for assessing whether the engraver was right- or left-handed, for ascertaining the direction and order of the engraved lines, and for deciding whether marks were made quickly or accumulated over a period of time. In the past, studies of prehistoric engravings on stone, bone, antler

of traces depending on the part of the tool used, its position and angle, the strength of the hand and the pressure applied⁵. He has claimed that some marks engraved on Stone Age artefacts were notations, accumulated slowly, and in a few cases were observational lunar notations.

Francesco d'Errico, at the Institut de Paléontologie Humaine, Paris, has carried out some fascinating experiments^{6,7} with particular reference to the series of parallel engraved lines found on some epipalaeolithic (azilian, about 10000–8000 BC) pebbles from France. He began by making 50 burins of flint and jasper, duplicating the morphology, technology and dimensions of tools characteristic of the late French upper palaeolithic and epipalaeolithic. With these, he marked 100 limestone pebbles of different shapes, sizes, textures and degrees of compactness. The lines were straight or



a, Extremities of experimental engraved lines (a) showing secondary marks (b) made by the same tool. b, Extremities of engraved lines (a) on azilian pebbles from Rochedane (France) showing secondary marks (b) identifying the use of a single tool. c, Demonstration of how secondary striations (b) are made by a burin at a curved pebble-edge. d, Engraved azilian pebble from Rochedane showing position of b.

and ivory focused primarily on the style and content of the markings. It is really only in the past 20 years that interest has turned to the technology of production — the properties of the materials and tools used, their influence on the artistic results, and the time and effort taken in making accurate copies.

Alexander Marshack⁴ turned the study of prehistoric engraved surfaces into a subject of scientific research rather than a simple offshoot of traditional artistic studies. He used the reflected-light stereoscopic microscope to follow the mechanics, micromorphology and 'ballistic trace' of each incision (its point of impact and subsequent path) and to identify marks made in different ways: as arcs, jabs and so on. He has also claimed to be able to detect changes of tool and of hand, whereas others have argued that, on small stone plaques at least, a single burin or engraving tool can produce a wide variety

of varying lengths, made with a single stroke or several uni- or bidirectional strokes, and beginning either in the centre or at the edge of the pebbles. In addition, several lines were made to intersect, forming various angles against the surface and the angle at which they were held, and used several pebbles as hammers and as retouchers to study the resulting wear on their surfaces.

The resolution that can be achieved with the scanning electron microscope reveals details that had not previously been seen on the experimentally marked surfaces. Engraving a line not only removes material from the pebble but also deforms its natural texture and creates compact areas. Each line displays a varying morphology throughout its length: the starting point comprises a crushed cavity, from which the groove proceeds like a comet's tail. The groove itself is a longitudinal zone of abrasion containing

1. Cava, R.J. et al. *Nature* **332**, 814–816 (1988).
2. Zasadzinski, J.F. et al. *Physica C* **158**, 519–524 (1989).
3. Loong, C.-K. et al. *Phys. Rev. Lett.* **62**, 2628–2631 (1989).
4. Reichardt, W. & Weber, W. *J. appl. Phys.* **26**, 1121–1122 (1987).
5. Mattheiss, L.F. & Hamann, D.R. *Phys. Rev.* **B28**, 4227–4241 (1983).
6. Shirai, M., Suzuki, N. & Motizuki, K. *J. Phys: Condens. Matter* **1**, 2939–2943 (1989).
7. Sato, H., Tajima, S., Takagi, H. & Uchida, S. *Nature* **338**, 241–243 (1989).
8. Weber, W., *Phys. Rev. Lett.* **58**, 1371–1374 (1987); *erratum* **58**, 2134 (1987).
9. Cohen, R.E., Pickett, W.E. & Krakauer, H. *Phys. Rev. Lett.* **62**, 831–834 (1989).
10. Guvitch, M. & Flory, A.T. *Phys. Rev. Lett.* **59**, 1337–1340 (1987).

areas where the granular and porous texture of the pebble has been exposed by removal of the surface; in some cases there are also parallel broken bands of extremely compact material, often with parallel striations running through them: the presence of these compact surfaces seems to be directly linked to the tools being used slowly or under great pressure.

There are two apparently valid criteria for assessing a tool's direction of movement. First, the compact areas are often 'scaly' at the edges or in the middle because of tiny superficial lacerations, and in the experimental engravings these scales were consistently raised in a direction opposite to that of the tool's trajectory. Second, the porous areas may be rippled or bear a succession of semicircular cavities with their convex surface facing the direction of movement. Similar scales and arcs occur in the abraded parallel grooves on pebbles used for retouching, again showing clearly the direction of movement.

At intersections of engraved lines it is often easy with a simple microscope or with plasticine imprints⁵ to see the order in which they were made. The scanning microscope makes this observation even more straightforward, as the later line removes any compact areas or striations in the earlier groove at the crossing point, whereas its own remain unbroken. In addition, the later groove often produces an irregular cavity at the intersection which also reveals the direction of its trajectory. In some cases, contact between two lines causes lateral slipping of the later groove at the crossing point, and the direction of the slip provides a further clue to the trajectory of the tool: the shift is downwards if the line is engraved from left to right, and to the left if the groove is made vertically downward.

The micromorphology of these incisions also reveals in which hand a tool was held: where an engraving is made from left to right by a right-handed person, the compact part of the abrasion occurs along the side of the groove that is closest to the experimenter. Where a right-hander makes a vertical groove downwards, the compact band occurs along its centre and left side.

D'Errico has produced definite criteria for identifying marks by the same tool: engraved lines are accompanied by minute secondary or 'parasite' striations caused by a partial, momentary contact between other parts of the tool with the pebble surface: the number, morphology and location of these fine marks constitute a unique 'tool signature'⁷ (*a* in the figure). It has even been possible to see the point in an incision where slight breakage of the tool has occurred, and the line has continued with an increase in surface contact and a change in the morphology of the incision⁸. The secondary marks are

particularly prevalent where lines are prolonged to a pebble's edge and the stone's curvature allows other parts of the tool to touch (*c* in the figure). Identical secondary striae in adjacent incisions imply the use of a single tool; because the striae are not a constant feature, but occur only where incisions were made in rapid succession, they allow the time when the engravings were made to be estimated.

D'Errico has compared these carefully documented observations with prehistoric specimens, mainly azilian pebbles from Rochedane, north-east France⁹ as well as specimens from other French sites. He looked at accurate resin replicas made by silicon elastomer impressions of their engraved surfaces in the microscope. He found the same marks at the starting points of lines, and found the same distinctive morphology at intersections, and the successions of semicircular cavities showing the direction of tool movement. On the other hand, compact areas are rare in the azilian lines and, where visible, have an altered surface clearly caused by post-depositional modification.

On those pebbles which have series of parallel engraved lines (*d* in the figure), the incisions were made from the centre to the edge, mostly from left to right by a right-hander. At the edges, the pattern of secondary striations was the same as that on the experimental specimens (*b* in the figure) and revealed that adjacent incisions had probably been made in rapid succession by the same tool, probably as a single operation.

This observation certainly suggests that the series of marks studied by d'Errico were made rapidly and are not, therefore, slowly accumulated notations. However, the strongest claims for prehistoric lunar notations have been made for notches on bone⁴, and it remains to be seen how far d'Errico's results are applicable to these; many of his morphological indicators appear similar to those described for butchering marks on bone¹⁰.

The extension of his method to purposeful incisions on organic materials will be the next stage of the debate. Although everyone agrees that notations may well have been made in prehistory, disagreement remains about whether their existence has yet been proved¹⁻³. □

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1. d'Errico, F. *Curr. Anth.* **30**, 117 (1989).
2. Marshack, A. *Curr. Anth.* (in the press).
3. d'Errico, F. *Curr. Anth.* (in the press).
4. Marshack, A. *The Roots of Civilization* (Weidenfeld and Nicolson, London, 1972).
5. Bahn, P.G. & Vertut, J. *Images of the Ice Age* (Windward, Leicester, 1988).
6. d'Errico, F. in *Scanning Electron Microscopy in Archaeology* (ed. Olsen, S.L.) 169 (Brit. Arch. Rep. Int. ser. 452, Oxford, 1988).
7. d'Errico, F. *C.R. Acad. Sci. Paris* **304**, sér. II, 761 (1987).
8. d'Errico, F. *L'Anthropologie* **92**, 101 (1988).
9. Thévenin, A. *Gallia Préhistoire* **26**, 139 (1983).
10. Bromage, T.G. & Boyde, A. *Am. J. Phys. Anth.* **65**, 359 (1984).

Wave travel

OCEAN waves are an appealing and dramatic source of power. Rather surprisingly, their energy is essentially rotational. While the crests of the waves move forwards, their troughs move backwards, putting the water surface into closed elliptical trajectories. A stable shoreline is not needed to exploit these relative motions.

And so Daedalus is designing a wave-powered ship. It deploys a set of moveable paddles like stabilizers, which are extended into the local wave motion when it is moving with the ship, but retracted during its return flow. The ship thus ratchets over the sea on that component of the wave motion that coincides with its heading. Since a ship of fair size will span several wavelengths of the local swell, there will at any time be two or three wave elements around it with the desired motion. The wave thrust should therefore be fairly steady, and could be further smoothed and augmented by additional banks of paddles under the water, coupling into the deeper parts of the wave cycle. In principle, the 'paddleship' could achieve a reasonable fraction of the wave velocity itself — perhaps ten or twenty knots in a lively sea.

Control is the big problem. Luckily an infrared wave-scanning device has already been developed to help ships cope with bad weather. This detects approaching waves so that a bank of transputers can calculate the best disposition of rudder and stabilizers to minimize their effect. With minor modifications, the same system could be adapted for the paddleship, calculating the paddle strategy that will extract most energy from each wave as it arrives. The ship will make its best headway directly into, or directly against, the waves; in a beam sea it may have to tack. It will steer by phasing the portside paddles against the starboard ones, making it wonderfully manoeuvrable: 180° of phase shift will spin it about its own centre. Intriguingly, it will leave a calm wake behind it: a 'wave-shadow' region of depleted energy.

Such a simple vessel only needs a small crew; indeed, it could be completely computerized and unmanned. Tugs will tow a paddleship out to sea, and abandon it to its navigational program. Navigating by the global-positioning satellite transmissions, it will steer steadily to its destination, possibly with subtle detours to exploit vigorous sea conditions detected by satellite imagery and relayed to it from shore. Its radar will warn it of collisional hazards, which it will easily outmanoeuvre. In due course it will turn up at its destination to be captured by tugs and towed into port. The whole operation will be so cheap that, especially for stable commodity cargoes, its haphazard timetable will not matter. But it may face human hazards like stowaways and pirates.

David Jones

Acid rain and eggshells

SIR—The Netherlands has the highest forest decline due to acid precipitation in Europe¹. Studies on the effects of acid rain have concentrated on soil acidification in relation to changes in soil nutrient status, tree growth, disappearance of ectomycorrhizal fungi and leaching of nitrate. Little information is available on the effects on higher trophic levels in forest ecosystems and there have been few studies on the effects of acid precipitation on bird life²⁻⁴.

Our long-term study on population processes in the great tit and other hole-nesting bird species, carried out in deciduous and coniferous forests on both nutrient-rich and nutrient-poor soils, provides an opportunity to analyse the effects of acid precipitation on parameters related to reproduction. In Buunderkamp forest, which is representative of the coniferous and mixed deciduous forests on poor sandy soils in the eastern part of the country, we ringed all resident great tits. Between 1983 and 1988, we collected detailed demographic data in a poor and a relatively rich part of the forest⁵. We found there had been a sharp increase in the proportion of eggs with no shell at all or with shells of such a poor quality that the embryo dried up during incubation because of excessive evaporation (see figure). The occurrence of this phenomenon increased in up to 57 per cent of territorial birds in the poor part and up to 40 per cent in the relatively rich part. The increase for non-territorial birds, which normally have a relatively short period of local residence, was smaller. Blue tit, coal tit, nuthatch and great spotted woodpecker also had problems with eggshell forma-

tion, in contrast to the pied flycatcher, a migrant species, the female of which starts egg laying shortly after arrival.

We found strong indications for a similar increase in other forests on poor podzolic soil. On the other hand, we observed no increase in the percentage of breeding pairs with eggshells of insuffi-

oak and red oak, suggest a close relationship between the Ca^{2+} concentrations of leaves and caterpillars (data not shown).

From the data on the Ca^{2+} status at various trophic levels (soil, tree leaves, caterpillars, eggshells) of the forest ecosystem on poor sandy soils in the Netherlands, a direct relationship with the acid rain seems very likely. The data do not suggest that the numbers of great tits in poor forests are already declining because

Calcium-ion concentrations in leaves and needles of various tree species

Tree species	Soil				
	Poor (<i>n</i> = 7)		Rich (<i>n</i> = 5)		
	$\bar{x}$	s.e.	$\bar{x}$	s.e.	
Birch (<i>Betula alba</i>)	118	4.7	132	24.8	NS
Beech (<i>Fagus silvatica</i>)	75	8.0	134	14.2	*
Scots pine(<i>Pinus silvestris</i>)	67	7.0	105	7.2	*
Pedunculate oak (<i>Quercus robur</i>)	67	7.0	106	7.0	*
Red oak (<i>Quercus rubra</i>)	64	8.5	87	15.2	NS

Calcium-ion concentration in mmol per kg dry matter. (Test: Wilcoxon, * $P \leq 0.01$).

cient quality in woods of clay or loam soil.

The inferior quality of eggshells — very thin, granular, porous, fragile and without coloured spots — is related to an insufficient deposition of calcium. As a consequence of acid precipitation, Ca^{2+} in the soil is replaced at the adsorption complex by hydrogen ions and the Al^{3+} levels increase as the pH of the soil decreases, and at ratios of $\text{Ca}^{2+}/\text{Al}^{3+}$ smaller than 1 the uptake of calcium is impaired⁶. In the podzolic soils where the birds were observed, we found ratios of 0.3. The reduced supply resulted in low Ca^{2+} levels in the leaves of several tree species (see table). The relatively few data on Ca^{2+} content of caterpillars — in spring and summer an important food source for hole-nesting species — that could be collected in 1988 on leaves of pedunculate

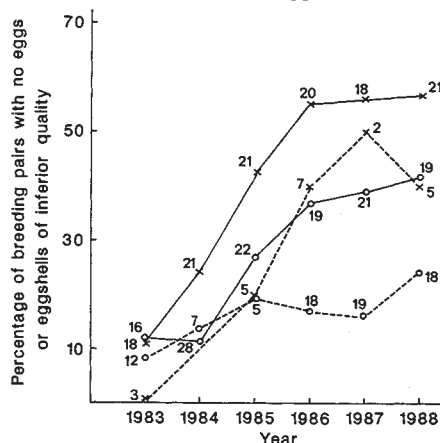
there is a large surplus in the production of young birds in the rich areas which by migration may compensate for an eventual shortage in poor forests. But our results imply that the effects of acid precipitation are becoming apparent at all trophic levels of forest communities.

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1. Results of the 1986 Forest Damage Survey in Europe, Usti nad Labem, Czechoslovakia, May 1987.
2. Erikson, M.O.G. *ICBP Tech. Publ.* 6, 183–189 (1987).
3. Schreiber, R.K. & Newman, J.R. *Conserv. Biol.* 2, 249 (1988).
4. Hölzinger, J. & Kroymann, B. *Ecol. Birds* 6, 203 (1984).
5. Drent, P.J. *Ardea* 75, 59–71 (1987).
6. Schneider, T. & Bresser, A.H.M. *Dutch Priority Programme on Acidification 00–04 Natn. Inst. Publ. Hlth env. Hygiene* (Bilthoven, 1987).



Increase in the percentage of territorial breeding pairs of the great tit with no eggs or eggshells of inferior quality. The data were collected in a poor part and a relatively rich part of the Buunderkamp mixed forest. Key: x — x territorial birds, poor part, x — x non-territorial birds, poor part (in 1984 this category was absent); o — o territorial birds, relatively rich part; o — o non-territorial birds, relatively rich part.

Mummy DNA fragment identified

SIR—The isolation of DNA fragments from a 2,400-year-old Egyptian mummy was reported by Pääbo¹. One clone, pMUM2:9, carried a segment of 3,400 base pairs (bp) — of which a 919-nucleotide fragment was sequenced — encompassing two members of the *Alu* family of repeats. We now report that 382 bp of the 5'-end of the first intron of HLA-DQA1 (DQ α), a human major histocompatibility complex (MHC) class II gene, is almost identical to a non-repetitive region of the mummy DNA. A very similar region also occurs in HLA-DQA2 (DQ α). The MHC class II α - and

β -chain genes encode heterodimeric surface proteins involved in the immune response. Genes encoding β -subunits are highly polymorphic, whereas α -chain genes exhibit much less variability, with the exception of the HLA-DQA1 gene. We have analysed dq-7 (Fig. 1), an *EcoRI/BamHI* subclone derived from H11A, a cosmid carrying the HLA-DQA1 and B1 genes². We characterized dq-7 by restriction mapping, hybridization and sequencing. A major transcription start site for the HLA-DQA1 gene³ has also been demonstrated.

At its 5'-end, dq-7 contains a repetitive



FIG. 1 Structure of the dq-7 subclone, with restriction sites. Vertical lines, *Alu* repeat; diagonal shading, A-rich; spotted region has 97% identity to a portion of mummy DNA (see text). The 5'-untranslated region/signal peptide (UT/SP) exon is also shown. Arrow, direction of transcription of the HLA-DQA1 gene; A and BA1 regulatory signals conserved among MHC class II genes.

Mummy: AATTCCTGA CAACCTT GAAGCTTTCGTAT 33
 dq-7: AATTTCCCTGAACAACTTTGAAGCTTTCGTAT 348

GTCTCTGTAGTAGATCTTGGGGTCTTCCATCAATTATAT 74
 GTCTCTGTAGTAGATCTTGGGGTCTTCCATCAATTATAT 307

ACTCTATAGATATTAATAA GTTCCCGTTTCTTCTCTCA 114
 ACTCTATAGATATTAATAAAGTGTCCCGTTTCTTCTCTCA 266

GACTTACTCACATTTCCACATGGGAAGTGGCACAGGTGGG 155
 GTCTTACTCACATTTCCACATGGGAAGTGGCACAGGTGGG 225

AGTGGGTAAAGAGTCCAGCAGGTGCAATGCCCTTCAACAAT 196
 AGTGGGTAAAGAGTCCAGCAGGTGCAATGCCCTTCAACAAT 184

CATTTTACCACATGGTCTCTACTCTCAGCTGCCTCAT 237
 CATTTTACCACATGGTCTCTACTCTCAGCTGCCTCAT 143

ATGTGTCACTCACAAA TAATCAAAATAAAA TGGGCATG 275
 ATGTGTCACTCACAAAATAATCAAAATAAAAATGGGCATG 102

TAGCTAAGCTTTGTAATAGTGAAACATGGATGCAATTG 316
 TAGCTAAGCTTTGTAATAGTGAAACATGGATGCAATTG 61

TTTTTACATATTTCTATTACAGGTATAGCTTCCATTTTTT 357
 TTTTACATATTTCTATTACAGGTATAGCTTCCATTTTTT 20

TTTAGCAAAAATAGGGATC 377
 TTAGCAAAAATAGGGATC 1

FIG. 2 Comparison of mummy DNA clone pMUM2:9 and dq-7. Lower-case letters mark non-identical bases; a few gaps have been introduced to maximize the alignment.

element, which turns out to be an *Alu* repeat, having ~80% identity with the *Alu* family average sequence and ~70% identity with the *Alu* member present in pMUM2:9 (nucleotides 494–782). Surprisingly, there is 97% identity between nucleotides 1–382 of dq-7 and nucleotides 1–376 of pMUM2:9 (Fig. 2), which excludes the *Alu*-like sequence of the latter. The sequence in the HLA-DQA2 gene equivalent to nucleotides 1–382 of dq-7 has about 87% identity to nucleotides 1–376 of pMUM2:9. Quantitative hybridization assays (calibrated with *Alu* and *L1* probes) and an exhaustive search in the computer catalogue (Microgenie, Beckman, 1987) demonstrate that the sequence is not a previously undescribed repetitive element.

One explanation of these findings may lie in the fact that pMUM2:9 was selected as a repetitive DNA-containing sequence by virtue of its hybridization with a *Bgl*II/*Sph*I genomic DNA fragment¹ that was derived from a HLA-DR β pseudogene⁴. This fragment, however, does not contain sequences (other than *Alu*) similar to either pMUM2:9 or to dq-7. Nevertheless, the *Bgl*II/*Sph*I probe may encompass a sequence characteristic of MHC class II genes that made possible the inadvertent isolation of HLA-DQA gene-related sequences from mummy DNA.

The high degree of conservation of a rather extended region of the first introns of the HLA-DQA1 gene of an ancient Egyptian individual and the DR4.4 cell line² strikingly contrasts with the frequency of polymorphism observed in modern population studies.

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1. Pääbo, S. *Nature* **314**, 644–645 (1985).
2. Okada, K. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3410 (1985).
3. Auffray, C. et al. *Immunogenetics* **26**, 63–73 (1987).
4. Larhammar, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1475 (1985).

Female choice

SIR—von Schantz *et al.*¹ report that female pheasants (*Phasianus colchicus*) choose males on the basis of the length of the male's spur in preference to other biometric variables or territory quality. They find no correlation between the quality of a male's territory and the number of females settling on it. This, however, does not necessarily mean that females do not assess and choose males on the basis of their territories; perhaps the cues females use have not been found or adequately measured.

von Schantz *et al.* base their conclusion on the assumption that their measure of territory quality is in fact what females are using to assess territories. To support this assumption, they demonstrate a statistically significant correlation between their index of territory quality and the number of chicks hatched. Their correlation coefficient is rather low ($r = 0.31$), however, which means that less than 10 per cent of the variance in chick number is explained by territory quality², suggesting their quality index is unreliable.

A second important point to their argument is an absence of a relationship between spur length and dominance in captive birds during the winter. Because male pheasants typically form winter flocks and then set up solitary territories during the breeding season³, the degree and nature of intrasexual competition is likely to be very different in the two seasons. It seems a great leap of faith to assume that dominance relationships of captive birds in one season will be an accurate predictor of their territory quality obtained when free living in the next season. Indeed, the only dominance reversals seen in one study³ occurred at the time that males were setting up territories.

von Schantz *et al.* tested their findings

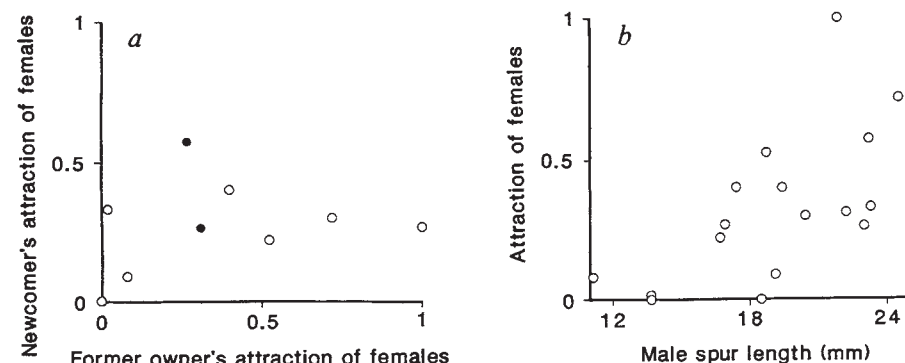
further by manipulating spur length, and find that long-spurred males have more females but are not more dominant. But again they fail to measure dominance during the critical time when territories are being established and females are choosing mates, and they do not assess the males' territories at all. It is hard to believe that spurs do not function in intra-sexual selection, because males use them for intrasexual combat when setting up territories⁴.

An alternative explanation for their results is that females are choosing males indirectly by their territories because territory quality has at least some effect on their reproductive success. Males, on the other hand, use their spurs in combat to obtain the best territories. This would also best explain the finding that females mating with long-spurred males rear the greatest number of chicks, because these females would be in the best areas. Given that spurs are used in combat, that territory quality is important to female reproductive success, and that female choice for territory quality is well known in other birds⁵, this hypothesis seems more likely.

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VON SCHANTZ *ET AL.* REPLY—In our study¹, we measured male dominance in the enclosure during March — the birds' last month in captivity. On the first week-day of April all birds were released and less than 2 weeks later most males had established territories (median=9 days after release, data from 1984–87, $n=93$). We believe that dominance measured in this way gives accurate data about the outcome of intrasexual conflicts uncorrelated to ownership of a given territory. This is important because we aimed to study the effects of male characteristics on male



a, Male attraction of females (measured as in Tables 1 and 3 in ref. 1) as a function of the former territory owner's attractiveness ($r = 0.23$, $P = 0.56$, $n = 9$). The sample is based on 9 cases, including 17 pheasant males, where the same territory was occupied by different males during successive breeding seasons. A territory is considered to be the same between 2 years if the overlap of the two estimated territories, as measured by the harmonic mean method⁴, is > 70% of their average area. One territory was occupied by three different males during three consecutive breeding seasons and is therefore represented twice in the sample (filled circles). Attraction of females is presented as the proportion of the highest estimate recorded during the particular year (1986–88). b, Male attraction of females as a function of the 17 territory owners' spur length ($r = 0.63$, $P = 0.007$, $n = 17$).

dominance and whether male dominance *per se* affected male mating success. Male dominance is probably strongly correlated to territory ownership³. Territorial changes are rare; we have not observed any enforced territorial take-overs in the study area.

Savalli takes the 'low', but significant, correlation ($r^2=0.10$) between female territory quality and the number of chicks hatched as an indication that our quality index is not valid. This conclusion seems premature. Data reported in ref. 6 reveal that independent variables explaining more than 10 per cent of female annual reproductive success in birds are rare.

Our new analysis, based on territories which had the same location, but were occupied by different males during successive years, reveals that the former territorial owner's attraction of females¹ did not predict that of the newcomer as would have been the case if male attractiveness was a function mainly of territory quality. Male spur length was a significant predictor of both former and newcoming territory

owner's attraction of females in this sample of males (see figure).

We agree that it is surprising that the variation in male territory quality does not seem to affect female choice significantly in our study¹. But the overwhelming majority of studies reporting that territory quality is the main factor for female choice are based on altricial species⁵ where the need for central place foraging makes territorial resources crucial for female reproductive success. Territory quality may be less variable for precocial birds because parents can freely lead their chicks to places where food is abundant.

Unfortunately, Savalli's arguments are based on a lack of expected correlations in our data¹ and when correlations occur, as opposed to what is "well known in other birds", the variables are "unreliable". We note that neither reference quoted by Savalli has quantified the relationship between harem size and territory quality or between male spur length and outcome of territorial combat. Although data do

not support Savalli's points, we do not exclude the possibility that female mate choice in pheasants is affected by territory quality or that male spur length affects intrasexual conflicts. Field experiments, rather than arguments, will tell whether these effects are of any significance.

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1. von Schantz, T. *et al. Nature* **337**, 166–169 (1989).
2. Snedecor, G.W. & Cochran, W.G. *Statistical Methods* 7th edn (Iowa State Univ. Press, Ames, 1980).
3. Collias, N.E. & Taber, R.D. *Condor* **53**, 265–275 (1951).
4. Glutz von Blotzheim, U.N. *Handbuch der Vögel Mitteleuropas* Vol. 5 (Akademische, Frankfurt, 1973).
5. Read, A.F. *Trends Ecol. Evol.* **1**, 85 (1986).
6. Clutton Brock, T.H. (ed.) *Reproductive Success* (University of Chicago Press, 1988).
7. Dixon, K.R. & Chapman, J.A. *Ecology* **61**, 1040 (1980).

Three's company for stereo viewing

SIR—Vance Tucker¹ clearly describes an under-appreciated ambiguity associated with stereo diagrams: a stereo-pair can give the proper three-dimensional image or a 'backwards' one depending on whether it is viewed with crossed or uncrossed visual axes. The standard arrangement for publication requires uncrossed visual axes as is appropriate for conventional stereoscopes. As Tucker points out, however, many people do not keep a stereoscope handy and use direct, 'naked-eye' viewing instead.

Unfortunately, as direct viewing is easier to learn using crossed visual axes², many people end up looking at backwards images. In the case of 'wire' models, the resulting front-to-back reversal simply changes the handedness, but in solid models it produces an unintelligible (pseudoscopic) image, as shown in the figure (which is taken from ref. 3 which contains a detailed description of the format together with a step-by-step method for learning direct viewing).

No arrangement of stereo-pairs can satisfy both methods of viewing but, as Tucker illustrates so nicely, three side-by-side diagrams is a universal format that can be viewed by any method. Why not publish all pictures in that format so that everyone can see a correct image using whatever means is at hand? The large page size of most journals would make this feasible; indeed, the space adjacent to stereo-pairs is usually blank. This triplet format has been used in both book³ and journal⁴ illustrations and is the standard format in the journal *Protein Engineering*.

For the triplet format it is important to note that the perceived depth differs with the method of viewing: uncrossed viewing gives rise to a larger percept with more apparent depth than the same figure viewed with crossed axes. To compensate, the individual images in the figure are rotated to different extents about their vertical axes. The pairs of images on the left (for uncrossed viewing) differ by a 4-degree rotation; the pairs on the right (for crossed

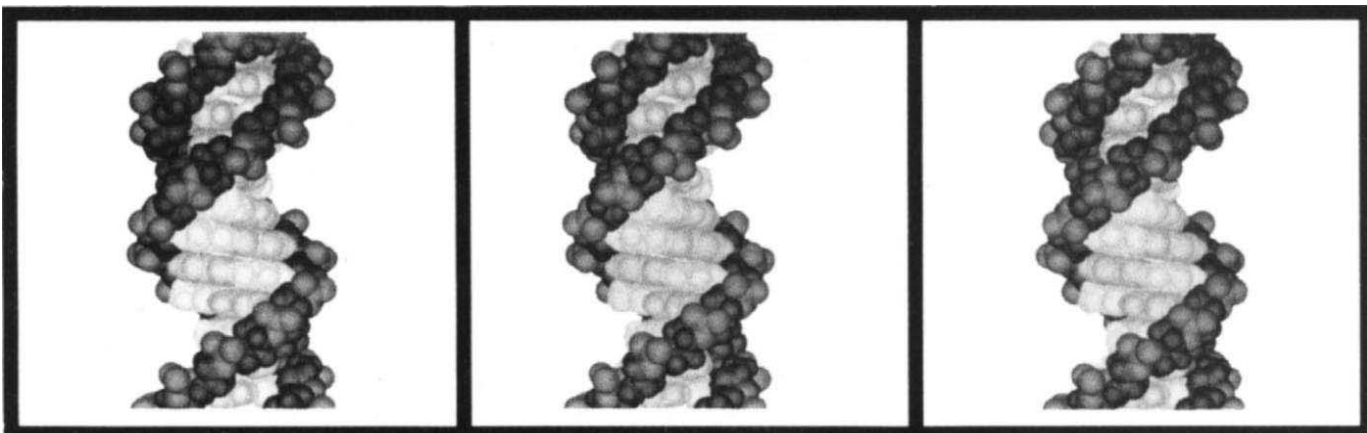
viewing) differ by an 8-degree rotation. The apparent depth in the two correct three-dimensional images is about the same.

Two additional—but inessential—features are incorporated into the stereo-triplet in the figure. First, a surrounding grid is included to make it easier to scan from image to image by direct viewing. Second, the central image is placed slightly to the left of centre so that the correct three-dimensional image appears to float just above the page, as defined by the grid. (Putting the adjacent pseudoscopic image below the plane of the page.) A universal stereo format that combines our technical and human capabilities might greatly improve the appreciation of three-dimensional structure in biology.

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1. Tucker, V. *Nature* **337**, 605 (1989).
2. Hamori, E., Broad, R. & Reed, C. *Perception* **11**, 297 (1982).
3. Wood, W.B. *et al. Biochemistry: A Problems Approach* (Benjamin/Cummings, Menlo Park, 1981).
4. Wilson, J.H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3641 (1979).



Lascar Volcano set to erupt?

SIR—Earlier this year, we showed how short-wavelength infrared observations from remote-sensing satellites can be used to monitor the radiant thermal flux from active volcanoes (L. Glaze *et al.* *Nature* **338**, 144; 1989), using Lascar volcano, north Chile as our example. We showed that the radiant thermal energy flux had decreased before a phreato-magmatic eruption in September 1986, and had subsequently increased greatly, doubling between October 1986 and November 1987, our last data point.

Reports from Paul King and his colleagues at Minsal, Toconao, Chile, show that a lava dome is now growing in the summit crater of Lascar. When first observed on an ascent of the volcano in February 1989, the lava dome had filled the summit crater to a depth of several tens of metres. The lava dome was absent when the crater was previously observed in January 1987, but a powerful thermal anomaly on the image we acquired in November 1987 suggests it may have been growing then. Although there are 50–60 major and potentially active volcanoes in the central Andes, many of them over 6,000 metres high, no other lava eruptions have been documented. Lascar is clearly in an unusually active condition, and warrants further monitoring.

Whereas the observation of this lava dome at Lascar emphasizes the value of remote-sensing techniques in monitoring remote volcanoes, we stress that there are many current limitations, not least of which is the 16-day repeat period of the Landsat satellite used in our studies and the vagaries in behaviour of individual volcanoes. Future generations of remote-sensing satellites, such as those forming part of NASA's Earth Observing System will offer much greater scope for volcanological studies. Detailed plans for these are already in hand. Initially, we believe that these techniques are valuable tools for monitoring volcanoes; prediction of the eruption of an individual volcano will remain difficult, ideally involving data from as many different sources as possible, both terrestrial and space-borne.

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Impacts and the origin of life

SIR—The point in time at which life could first appear on Earth through chemical evolution within a time interval between impact events was considered by K.A. Maher and D.J. Stevenson (*Nature* **331**, 612–614; 1988) for assumed chemical or prebiotic evolution times of 10^5 – 10^7 years. We would like to point out an error that leads to an incorrect estimate of this time. In Maher and Stevenson's model, the number of events per unit time was derived for each of four classes of impact effects that were considered to be adverse to the evolutionary process. The reciprocal of these values are the time periods between the impact events: T_c is the time required to obliterate the surface by crater overlap; T_e is the time to cover the surface by 3 metres of ejecta; T_w is the time between impacts that frustrate life on a global scale by causing climatic disruption, sterilization of the surface or sterilization of the entire planet; and T_b is the time taken to obliterate the ocean bottom by crater overlap. These time periods were given by

$$T_c = 1.0 \times 10^{11} [f(t)^{-1}] [(D_{\max}/1 \text{ km})^{0.2} - (D_{\min}/1 \text{ km})^{0.2}]^{-1} \text{ yr} \quad (1)$$

$$T_e = 1.0 \times 10^{11} [f(t)^{-1}] [(D_{e,\max}/1 \text{ km})^{0.51} - (D_{e,\min}/1 \text{ km})^{0.51}]^{-1} \text{ yr} \quad (2)$$

$$T_w = 250 [f(t)^{-1}] [(D_{\min}/1 \text{ km})^{2.8} \text{ yr}] \quad (3)$$

$$T_b = 5.0 \times 10^{10} [f(t)^{-1}] [(D_{\max}/1 \text{ km})^{0.2} - (D_{\min}/1 \text{ km})^{0.2}]^{-1} \text{ yr} \quad (4)$$

The proportionality constants in these equations consist of the products of various constants associated with impact parameters and a crater-production constant k corresponding to eight times the crater production rate on the Moon. The function $f(t)$ is an exponential decay function that allows for the changing flux of impactors early in planetary history. This function is $f(t) = 1 + \exp[(t-t_0)/T]$, where t is the time measured backwards from the present, $t_0 = 3,400$ million years and $T = 70$ million years. $D_{\min}$ and $D_{\max}$ are the minimum and maximum diameters of crater sizes contributing to the impact effect being considered.

In deriving equation (3), Maher and Stevenson consider the time interval between impacts that are just large enough to frustrate life on a global scale. For any particular global-impact effect, however, a range of crater diameters, from those just large enough to cause the effect up to the size just large enough to cause the next higher level of destruction, should have been used in determining the expected time interval between these events. That is, the time interval between events should be calculated from the product of the surface area of the Earth and the integral of the incremental number density evalua-

ted over the range of crater sizes capable of causing that effect.

We obtain

$$T_w = 1,400 f(t)^{-1} [(1 \text{ km}/D_{\min})^{1.8} - (1 \text{ km}/D_{\max})^{1.8}]^{-1} \quad (5)$$

for the time between impacts that frustrate life on a global scale. We used this equation, rather than equation (3), to calculate the time intervals between global events. $D_{\min}$ and $D_{\max}$ are 55 and 265 km, 265 and 850 km, and 850 and 2,600 km, for climatic disturbances, surface sterilization and planetary sterilization, respectively.

When the time period between events disruptive to chemical and early biotic evolution became greater than the time required to evolve life, life could first have appeared on Earth. Maher and Stevenson assumed that life formed in 10^5 – 10^7 years and calculated the point in time at which impact events became infrequent enough for life to form. With our revised equation (5) instead of (3), life could first appear at times more recent than those reported by Maher and Stevenson. For example, if large single events with energy of the order of 10^{34} erg, assumed to have been capable of 'sterilizing' the entire planet, were required to frustrate development of life, Maher and Stevenson find that life could have first originated between 4,100 and 4,300 million years ago, whereas we calculate this time interval to be 3,700–4,000 million years ago. Also, using equation (5) instead of equation (3) changes the sequence of times at which life can first appear uninterrupted by each of the impact effects. Using equation (3), for example, life appears more recently if it can be interrupted by crater obliteration rather than by planetary sterilization, whereas, using equation (5), the reverse is true. This reordering of the sequence of times at which life can first appear constitutes a significant difference between the results of Maher and Stevenson and those presented here.

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Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □

Mistaking a claim

Derek Fordham

The Arctic Grail: The Quest for the North West Passage and the North Pole, 1818–1909. By Pierre Berton. Viking: 1989. Pp.672. £16.95, \$24.95.

The Noose of Laurels: The Discovery of the North Pole*. By Wally Herbert. Hodder & Stoughton: 1989. Pp.395. £16.95.

"THE Pole at last!" Peary proclaimed standing at least 90 miles from that point. 'The Big Nail', as the Eskimos called the Pole, was the goal for which he had unsuccessfully striven for over 20 years and it had eluded him again on his last attempt.

Pierre Berton's entertaining and at times provocative book puts a perspective on the relentless struggle through the nineteenth and early twentieth centuries for knowledge of the polar regions, in particular the North West Passage and the North Pole. The world's ignorance of the Arctic at that time was paralleled by that of the Eskimos of north-west Greenland, who met Ross in 1818 and asked him, "Where do you come from?". The answer, "The south", was inconceivable to them; "All our ice goes there!"

The names of all the Arctic pioneers are here — Parry, McIntock, Franklin, Hall, Tyson, Andree, Amundsen, Peary and Cook — and Berton has put his emphasis on the fascinating personalities of these men, on their strengths and weaknesses, ambitions and passions as much as on their achievements. He has painted a stirring picture of a world of exploration dominated by diverse and remarkable characters. They were united by their consuming desire to discover a passage that perhaps did not exist and would have no commercial value, or to reach an unidentifiable point at the top of the world that had little or no scientific significance.

Commander Robert E. Peary was one of the protagonists in this long-drawn-out drama, and for many years those who have travelled by dog sledge on the polar pack ice have known that some of his claims for daily mileages achieved could not be true. No explorers achieved such distances before Peary and none has since. Both Berton and Herbert find his claims for his final dashes, in 1906 for the furthest north, and in 1909 for the Pole itself, to be unacceptable, in view both of his previous much

more modest performances when in the company of those capable of checking his records, and of the careless manner in which his navigational observations were made and recorded.

Wally Herbert's comprehensive and scholarly study is based on examination of navigational documents of Peary's not available to earlier chroniclers of the life of this bold, impassioned and eminently dislikeable man — a man whose love and affection for his family were over-ridden by his ruthless, consuming desire for the

fame and fortune that he believed priority at the Pole would attract. The book, which will surely stand as the definitive work on this controversy, has the curious sub-title, *The Discovery of the North Pole*; curious, because Herbert establishes beyond doubt that its principal subjects, Peary and Dr Frederick Cook, were almost certainly never anywhere near the Pole.

Few previous expeditions exploited the Arctic and its people in as systematic and ruthless a manner as Peary's. His dealings with the Eskimos, whose contact with his trade goods brought them out of the Stone Age in the 23 years during which he was a regular visitor, did not earn him universal acclaim — particularly not from those people he cajoled and bribed into pressing on across the Arctic pack ice out of sight of their land, and then got to supply him with walrus and narwhal ivory to support a profitable business back home. He must, however, be given credit for having the foresight to adopt native methods of sledging, and to a remarkable degree more than other explorers he understood the value of dogs. Others had been forced on occasion to eat their animals. Peary *planned* to eat his and by doing so increased the range of his sledge parties considerably.

From *The Noose of Laurels*

Peary started on what he knew was to be his last attempt on the Pole in 1909, following his expedition of 1906 when he claimed the record for having travelled furthest north. On that expedition he had claimed mileages well above his previous achievements, but only during the later stages of the journey when he was accompanied only by Eskimos and his negro servant Matthew Henson, none of whom were able to confirm or contradict his navigational observations.

The pattern was repeated in 1909. Following Peary's order for the return of the supporting sledge parties, which included a disappointed Bob Bartlett, an experienced sea captain and navigator, the claimed daily mileages increased from 12 to 43, not counting detours which Peary had previously put at an additional 25 per cent. Failure was of course unthinkable: nobody wanted another 'almost' or 'furthest' record, and if he failed to reach the Pole bankruptcy threatened. As if that was not enough, this time Peary had a rival: his erstwhile friend Frederick Cook had set off from Greenland a year earlier with the object of crossing Ellesmere Island and reaching the Pole by



Robert E. Peary — "bold, impassioned and eminently dislikeable".

way of Nansen Sound. Cook had not been heard of since. Thus the stage was set for the controversy which was to follow the two men to their graves and beyond.

Peary returned from his furthest north, almost certainly nowhere near the Pole, and reached the north coast of Ellesmere Island only five days behind Bartlett. Ootah, one of his Eskimos, remarked "The devil must be asleep or having trouble with his wife or we should never have come back so easily". Peary appears to have wrestled with his conscience for some days after returning to his ship, frozen into the coastal ice, before announcing that he had reached the Pole. The gap between truth and ambition had been crossed. Peary had donned his Noose of Laurels.

On his return to civilization, Peary discovered that Cook had claimed to have reached the Pole a whole year earlier. Cook was a gentler and more immediately likeable character; one can feel compassion for him, the Prince of Losers, so casual a man that he left the instruments and records of his polar journey in Greenland. He had previously made a claim, later judged to be false, to have been the first to ascend Mount McKinley in Alaska and this fact, together with the totally self-contained logistics of his polar journey, became the basis for the controversy that has raged for the past 80 years over who was first at the Pole. Cook's claim was eventually discredited, one reason being lack of conclusive proof. This was precisely what Peary could not produce either, but the insistent voices of the press and geographical societies of the time seemed to concur: if Cook had not reached the Pole, Peary must have. But did he?

"There is nothing worth living for but to have one's name inscribed on the Arctic chart" wrote Tennyson of the search for Franklin. Along with distillers, brewers and merchant princes, Peary achieved just that. But today his own documents seem to have revealed that his name was inscribed in the wrong place and The National Geographic Society, his original and faithful sponsor, is now mounting an investigation into those of his claims that have excited so much scepticism.

For Peary, out on the pack ice at the end of his lines of support, miles from the Pole and unable to face further attempts to reach it, the risk of such an investigation could not at the time have been envisaged. But he would surely have taken such a risk in order to be able to make the claim by which he became famous and by which, over the years, his Noose of Laurels tightened — "The Pole at Last!" □

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* To be published in the United States by Atheneum.

The right stuff for the future

John Wood

Encyclopedia of Materials Science and Engineering: Supplementary Volume 1.

Edited by Robert W. Cahn. Pergamon, UK/ MIT Press, USA: 1988/1989. Pp. 653. £160, \$295.

I HAVE always wondered who bought copies of the yearly additions to general knowledge encyclopaedias, since by their very nature such volumes are for dipping into rather than reading in a coherent way. At least they have the advantage of an historical focal point, and its absence in this first supplement to the eight-volume *Encyclopedia of Materials Science and Engineering* means it is difficult to review the book in isolation. Editorial control is now in the hands of Professor Robert Cahn, who states that the objectives of the supplement are to describe new discoveries, update previous contributions and to remedy omissions. I also suspect that some of the articles (for example those on creep of metals and long-range order) probably did not meet the deadline for the main series. Professor Cahn's cosmopolitan influence can be detected in that he has sought authors from all points of the globe, who as well as giving accounts of their topic also demonstrate the rich variety of ways in which materials are perceived and understood by different nations.

The preface gives little clue as to how the present articles were actually selected, and indeed much of it is devoted to a description of how the whole encyclopaedia will be reformatted into concise volumes on specific topics (advanced ceramic materials, mineral resources and so on). The emphasis on the new initiative seems in part to underline the problems of finding articles relevant for a specific situation (highlighted in the earlier review in *Nature* 324, 26; 1988), which is now compounded by having to refer to the two subject indexes. In some senses we see a tortured debate on the true identity of materials science and engineering through these pages, so much so that the editor believes it necessary to justify the inclusion of articles on water/ice and printing inks. As it happens these sections are extremely interesting, and they demonstrate that there is a marked similarity in processing and performance indicators that cover almost all materials whether natural or artificial.

Indeed, the thesis of this review is that the unity of materials science and engineering lies more in their application than in the fundamental scientific principles behind complex systems (which are still,

in many cases, ill-understood). Thus it is the contributions on dental materials, paper, water for reactors, wound-dressing materials and so on that pull the subject together, rather than those (well-written as many of them are) on numerical modelling of solidification and various structural analytical techniques. Those who have already invested in the main encyclopaedia will not be disappointed by the supplementary volume. As a one-off purchase the book is a doubtful investment, and it will be worth waiting for the concise editions to appear if you want to take advantage of the many excellent contributions.

The encyclopaedia in its many forms does raise the problem of the identity and purpose of materials science and engineering. Much has been said about the 'materials revolution' — 'materials 2000' and the like. These initiatives are often so much boardroom hyperbole, and herein lies the dilemma for materials engineers. Do they work on upfront technologies, knowing that much of industry still does not adequately understand the materials and processes that already exist? The problem is that the design engineer or specifier finds it difficult to reverse the thinking process and change from a materials-limited to a materials-led philosophy. Vapour phase, molecular beam and microwave processing techniques now allow extremely fine control of what atom sits where. If you want a diamond coating on a wooden substrate filled with conducting polymer, then you only need to ask. As P.R. Bridenbaugh has remarked, "we are in a subtle but profound change in leadership within the materials industry ... from the traditional materials companies to product manufacturers" (*MRS Bulletin* 13, 19; 1988). Large companies are already setting the pace, and the materials expert will increasingly be part of a product team trying to meet design and market objectives. Viewed in this light, the encyclopaedia and its supplements stand as the pinnacle of the old order heralding the new. The rejigging of articles into the concise volumes on specific materials has missed this change of requirement; although these volumes will no doubt bump up the royalties for many worthy contributors, to my mind they will not meet the main need.

What then does the next decade hold? Undoubtedly the emphasis will be on custom-made structures, in which individual elements are blended together in a composite to match performance specifications consistent with minimal wastage and cost. Increasing advantage will be taken of surface treatments to ensure environmental compatibility. There are likely to be gains in semiconductors, polymers, optical materials, ceramics and diamond-coating research, but in economic terms the micromanipulation of structures, for example by spray deposi-

tion (Osprey process) for the production of bulk engineering components will be those that will mark the 1990s.

It would be foolish to bet on any specific material, because the future will see the consolidation and integration of many current materials and technologies. If I were to stick my neck out, I think that there will be a new era for wood and other biological materials. Timber produced from microwave-treated wood has recently opened up a fresh vista for structural engineers. Silk and artificially grown cartilage are also areas where lateral thinking and interdisciplinary research are beginning to pay dividends. We can also hope to see materials education turn from a frightened remnant based on the steel

industry to take a central role in the total design and production sequence. This may be despite the reactionary drag of the professional engineering institutions. There is a need for a coherent language that links the possibilities of scientific curiosity with the reality of engineering products. That link is to be found in the materials specialist, and it is time for the inferiority complex of the past two decades to be replaced by an optimism and sense of immediacy that the subject really deserves. □

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Special effects

F. Moriarty

Ecotoxicology: Problems and Approaches.

Edited by Simon A. Levin, Mark A. Harwell, John R. Kelly and Kenneth D. Kimball. Springer-Verlag: 1989. Pp. 547. DM90, £29.50, \$49.

ECOTOXICOLOGISTS attempt to study the effects of chemicals on ecosystems. In the early days, about 25 years ago, we struggled to reject the use of LD₅₀ tests and related measures, and to find more relevant ways of assessment. There was an important dichotomy of views in the underlying ecological science at that time. Clements had stated in 1916 that communities of interacting populations are organic entities comparable to individual organisms. Although Ramensky had published the first opposition as early as 1924, this view was widely accepted for the next 60 years. It is probably fair to say that the concept now has few adherents, but it still exerts a malign, usually covert, influence on the development of ecotoxicology.

In the second chapter of the book under review, J. R. Kelly and M. A. Harwell set the tone for much that follows by arguing at length that ecosystems are not supra-organisms and therefore one cannot expect to find one single or a few simple indicators of effects of exposures to chemicals. In particular the idea that functional indicators, such as rates of energy and of nutrient flow, are preferable to structural indicators, such as population size, is finally put to rest by several contributors. Kelly and Harwell stress that the choice of indicators of effects is a value judgement: in their terms, the choice should be "of relevance to issues of concern to humans". This statement deserves emphasis, particularly for what might be termed pure conservation interests — reasons why it is desirable to control impacts on wildlife are often fudged and obscured.

One might hope, after that start, for detailed examination of suitable indicators, but the opportunity is largely missed. Thus an account of genetic resistance to cadmium in one population of the oligochaete *Limnodrilus hoffmeisteri* concludes with the important, though unoriginal, point that the occurrence of resistance may be an effective indicator of effects on ecosystems, yet ignores the wealth of relevant material on this topic. One useful chapter describes the ecotoxicological requirements of seven pieces of legislation in the United States, including ToSCA (Toxic Substances Control Act), but with no accompanying critique. The next, final, chapter draws the obvious conclusion that we need therefore to monitor the permitted uses of chemicals, and need to be able to modify decisions in the light of experience. Despite the importance of monitoring, there is only one short chapter on the topic, and it is dominated, like many others, by a preoccupation with abstract concepts, in this instance control theory and decision analysis.

The book is not easy to use. The index is of very little help, sometimes with tens of page references per topic (58 for 'effects'). The writing is frequently ponderous, and there is considerable repetition between chapters. But those whose thinking is still influenced by the idea of a community as a supra-organism, or who are looking for an account of relevant US legislation, will find it respectively instructive or useful. □

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● Newly published by CRC Press is *Practical Handbook of Environmental Control*, edited by Conrad P. Straub, a reference work intended both for students and professionals. The material, presented largely in tabular form, is divided into sections on air, water sources and quality, wastewater, solid wastes, and institutions. Price is \$52.95, £32 (in Britain the book is distributed by Wolfe Medical, London).

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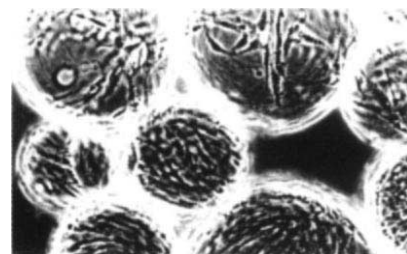
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Order and the square snail

Stephen Jay Gould

Games, Sex and Evolution. By John Maynard Smith. *Harvester-Wheatsheaf*: 1988. Pp.264. £16.95, \$30.

GREAT popular writing about the natural world may be allocated to one of two lineages, which I shall call franciscan and galilean. The franciscan lineage — from the eponymous St Francis himself, to Henry David Thoreau, to Loren Eiseley in our day — exalts nature in poetic terms, and works for empathy by resonance with the emotions. The galilean lineage may go back to Aristotle, but I designate as its standard-bearer the man who chose to write his two great treatises in the Italian vernacular (not the Latin of church and university), and as dialogues between a teacher and his students. This lineage, while not insensible to aesthetic beauty, emphasizes the loveliness of intellectual solutions, and views natural objects more as doors to understanding than portals to the soul.

Both lineages have their dangers and empty caricatures: overblown language (too often about sunsets) for the franciscans, pedantry and perverting simplification for the galileans. The great galileans, from the founder himself, to T.H. Huxley, to P.B. Medawar in our times, win their laurels for two reasons: because they understand the process of science so intimately (almost always because they practise the art as a profession, and do not only write as journalistic spectators), and because they know that clear, jargon-free prose need not simplify concepts, and that much of the beauty of science lies in its accessible intricacy and ambiguity.

J.B.S. Haldane, the mentor of John Maynard Smith, was the greatest galilean of his generation. He was also, in his technical work, Britain's leading evolutionary theorist. Maynard Smith, Emeritus Professor of Biology at the University of Sussex, succeeded Haldane in this technical arena, and is our foremost darwinian theorist today. In *Games, Sex and Evolution*, he conveys this work to a wider audience, and proves himself a worthy successor of Haldane in the field of galilean popularization as well.

All but one or two of the 28 pieces in this book have been published before, more than two-thirds of them during this decade, and more than half as book reviews. I cannot gainsay the genre of collecting such items into books, for I have dabbled in it myself. But two major dangers lurk, neither entirely avoided by Maynard Smith: obsolescence and repetition. For the first, most books quickly sink

without issue or new edition, and their epiphenomena (including reviews) die with them. To avoid obsolescence, one must do something slightly unfair to the author — use the existence of his book as an excuse to write an essay on something general and enduring (and then purge most parochial passages about the book when you republish the review). Maynard Smith comes close, but his legendary fairness has forbidden the necessary ruthlessness, and too much that is too ephemeral remains.

To avoid repetition, one must edit with a vengeance, another trait lacking in this kindest of men (apparently even towards his own literary children). No one — not Bach, not Handel, not the Lord Himself (or whoever wrote the Good Book) — can avoid telling the same story more than once in a long series of pieces on similar topics. But some redundancies should be eliminated on compilation. One, or even two, of Maynard Smith's lucid explanations of Fisher's theory on sex ratio would have sufficed.

Maynard Smith was originally trained as an engineer, and his mathematical skills far outstrip those of most colleagues in his adopted profession. He knows the limits of his methods, but he loves and dwells upon the power of simple mathematical models, both to pick out recurrent themes and causes amidst the world's complexity, and to suggest connections where disparate subject matter obscures a unity of underlying structure. The title of this book reads like a broad catch-all. But it really marks the doorway to a *roman-à-clef*, for the three subjects mark Maynard Smith's most successful application of his mathematical proclivities: his work on the vexatious problem of the evolution of sex (a frightful puzzle in Darwin's world, where organisms struggle only for personal reproductive success and should not dilute their genetic heritage by half in a sexual offspring, when asexual reproduction passes all parental genes to the next generation); his recognition that game theory illuminates much of darwinism, leading to his seminal formulation of ESS (evolutionarily stable strategy) theory; and his critique, as a mathematically minded and unrepentant strict darwinian, of an array of challenges proposed by people who study evolution on other scales (including this reviewer, who greatly values the criticism because it rests upon the two features, alas so rarely found, that characterize all fruitful and honourable intellectual debate — understanding an opponent, and taking his views seriously).

I appreciate both the elegance and power of Maynard Smith's world, a place ordered and dominated by natural selection working in Darwin's favoured mode — on organisms, for the passage of genes to future generations, and leading by struggle to pervasive adaptation. This tidy

world of small scale (where Maynard Smith's favourite styles of mathematics apply well) then yields, by smooth extrapolation across all domains of space and time, the pageant of evolution. Maynard Smith allows a plurality of other effects, but confines them to so insignificant a relative frequency that they just don't matter in the totality of his ordered world. Thus, species selection exists and may promote non-adaptive trends in phenotypes by hitch-hiking of numerous features on high speciation rates, but the process is "a very weak one" compared with conventional selection on organisms. Neutralism may be important, but only in a genetic domain below expression in phenotypes — and phenotypes are regulated by the "evolution of adaptations [as] the result of natural selection, and it is adaptation which is the most striking feature of the living world" (p.188). Side consequences and exaptations may produce striking features, but so long as selection triggers the chain, selection shall be the proper centre of explanation.

In my view, Maynard Smith's tidy world exists, but only in a restricted corner of totality, not as a model for everything. Nature is much messier — more imbued with the unpredictable contingencies of history; more replete with important products of selection working both below and above (genes and species) the conventional darwinian emphasis on organisms; more infested in vital ways with non-adaptive side consequences that can overwhelm any trigger of selection. (I don't for a minute accept Maynard Smith's assertion, made on p.82, that "our higher faculties, such as our ability to construct mathematical arguments, or to have religious experiences, [require] an explanation in terms of natural selection". Natural selection may have enlarged our brains for other reasons, but most of what we do with our mentality must arise as side consequences of the flexibility and power of the organization itself.) Maynard Smith tells us that "there cannot be gastropods with square shells" (p.142) — and he's right under the log spiral model of growth. But few shells grow as an ideal log spiral, and I have just finished a technical article on *Cerion alberti*, the square land snail of Cuba.

The world is not intractable, only gorgeously complex. Galilean rationalism need not cede to franciscan wonder. The American cub scout oath requires members to "be square [meaning honourable in American jargon] and obey the law of the pack". The world can have square snails (indeed it does!) and ordered behaviour — but the domains of evolutionary order extend far beyond the strict darwinian world of natural selection among organisms. □

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Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor

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The sequence of 5,037 amino acids composing the ryanodine receptor from rabbit skeletal muscle sarcoplasmic reticulum has been deduced by cloning and sequencing the complementary DNA. The predicted structure suggests that the calcium release channel activity resides in the C-terminal region of the receptor molecule, whereas the remaining portion constitutes the 'foot' structure spanning the junctional gap between the sarcoplasmic reticulum and the transverse tubule.

CONTRACTION of skeletal muscle is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum (SR) following depolarization of transverse tubules (T-tubules)^{1,2}. It has been suggested that the communication between T-tubules and SR occurs at the triad junction where arrays of electron-dense projections called 'foot' structures are interposed³. The use of ryanodine, an alkaloid that binds to the calcium-release channel in junctional SR and modulates its activity, has recently allowed the purification of the ryanodine receptor with a monomeric relative molecular mass (M_r) of ~400,000–450,000 (400K–450K)^{4–6}. The purified receptor protein has been morphologically identified with the 'foot' structure^{4,6,7} and has also been shown to function as a calcium-release channel when reconstituted into planar lipid bilayers^{5,6,8}. The channel properties and subcellular distribution of the ryanodine receptor suggest its involvement in the SR Ca^{2+} release that occurs during excitation-contraction (E-C) coupling.

We have now deduced the complete amino-acid sequence of the ryanodine receptor from rabbit skeletal muscle SR by cloning and sequencing DNA complementary to its messenger RNA. The receptor protein, consisting of 5,037 amino-acid residues, has four putative transmembrane segments in its C-terminal tenth. The remaining portion is assigned to the cytoplasmic side of the SR membrane. The cytoplasmic region preceding and close to the first transmembrane segment contains candidates for binding sites for modulators of the calcium-release channel. These observations suggest that the ryanodine receptor molecule consists of two main parts, that is, the C-terminal channel region and the large cytoplasmic region that apparently corresponds to the foot structure. We also show that expression of the cloned cDNA in Chinese hamster ovary (CHO) cells yields a protein indistinguishable from the ryanodine receptor in immunoreactivity, molecular size and ryanodine-binding properties.

DNA cloning

The ryanodine receptor from rabbit skeletal muscle SR was purified to essential homogeneity by the procedure of Inui *et*

*al.*⁴, followed by gel permeation HPLC under denaturing conditions (Fig. 1a). The purified preparation was subjected to trypsin digestion or to cyanogen bromide cleavage followed by lysyl endopeptidase digestion. The resulting peptides were fractionated by reverse-phase HPLC (Fig. 1b, c) and analysed for amino-acid sequence. Two synthetic oligodeoxyribonucleotide probes, prepared on the basis of partial amino-acid sequences of the tryptic peptide T2, were used to screen a cDNA library derived from rabbit skeletal muscle poly(A)⁺ RNA. The initial clone thus isolated was used as probe for cloning adjacent cDNA sequences and such procedures were repeated successively (see Fig. 2 legend).

To locate the mRNA start site, we cloned and sequenced rabbit genomic DNA corresponding to the 5'-terminal region of the cDNA (see Fig. 2 legend). *S*₁ nuclease mapping⁹ using the genomic *Ap*I(–354)/*Sma*I(–22) fragment and rabbit skeletal muscle poly(A)⁺ RNA showed that a band corresponding to 115–121 nucleotides was protected; restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5'-terminal nucleotide generated by cleavage (for nucleotide numbers, see Fig. 2). Primer extension experiments with the *Hpa*II(–112)/*Sma*I(–22) fragment or the *Sma*I(–22)/*Pst*I(69) fragment derived from the cDNA yielded major products of ~115 and ~200 nucleotides, respectively. On the basis of these results and of the fact that eukaryotic mRNAs generally start with an A residue¹⁰, the start site of the ryanodine receptor mRNA was tentatively assigned to the A residue –138 (Fig. 2). The genomic sequence corresponding to residues –131 to 48 of the cDNA is uninterrupted. There are two GC box sequences (residues –242 to –237 and –186 to –181) and a CAAT box sequence (residues –201 to –197) upstream of the mRNA start site, but no TATA box is found (see refs 10, 11). The 5'-noncoding region of the cDNA contains two copies of the sequence CCCCAGCC (residues –90 to –83 and –79 to –72) whose complementary sequence GGCTGGGG is found in the 5'-flanking region and an intron of the rabbit skeletal muscle Ca^{2+} -ATPase gene¹² and in the 5'-noncoding region (43 nucleotides upstream from the translational initiation site) of the cDNA encoding the 140K polypeptide associated with the rabbit skeletal muscle dihydropyridine receptor¹³. This motif might represent a skeletal muscle-specific enhancer element.

Protein structure

Sequence analysis of the cDNA reveals an open reading frame that encodes a sequence of 5,037 amino acids (Fig. 2). All the seventeen partial amino-acid sequences determined (see Fig. 1 legend) are encoded by the cDNA in the same reading frame. The calculated M_r of the rabbit skeletal muscle ryanodine receptor is 565,223 (including the initiating methionine), in reasonable agreement with the values estimated by SDS-polyacrylamide gel electrophoresis^{5,6} and electron-microscopic studies¹⁴. The

nucleotide sequence surrounding the ATG triplet encoding the initiating methionine agrees with the favoured sequence that flanks functional initiation codons in eukaryotic mRNAs¹⁵. The 3'-noncoding region of the cDNA is 119 nucleotides long (excluding the poly(dA) tract); the polyadenylation signal AATAAA (see ref. 10) is found 20 nucleotides upstream from the poly(dA) tract. The size of the rabbit skeletal muscle ryanodine receptor mRNA was estimated by blot hybridization analysis¹⁶ of poly(A)⁺ RNA to be ~16 kilobases, in agreement with our assignment of the mRNA start site and the polyadenylation site.

The deduced amino-acid sequence of the ryanodine receptor was analysed for local hydropathicity¹⁷ and predicted secondary structure¹⁸ (Fig. 3). The receptor molecule has four hydro-

phobic segments with predicted secondary structure in its C-terminal tenth. These segments (referred to as M1, M2, M3 and M4; overlined in Fig. 2), each comprising about 20 amino-acid residues, presumably represent transmembrane α -helices. The ryanodine receptor does not possess a hydrophobic amino-terminal sequence indicative of the signal sequence. This may indicate that the N-terminus is located on the cytoplasmic side of the SR membrane¹⁹. Thus the portion preceding segment M1, which constitutes nine-tenths of the receptor molecule, is assigned to the cytoplasmic side.

The calcium-release channel is activated or inhibited by various modulators^{1,2,20}, including Ca^{2+} , adenine nucleotides, caffeine, calmodulin and polycationic compounds. There are regions that bear some resemblance to the EF-hand²¹, for

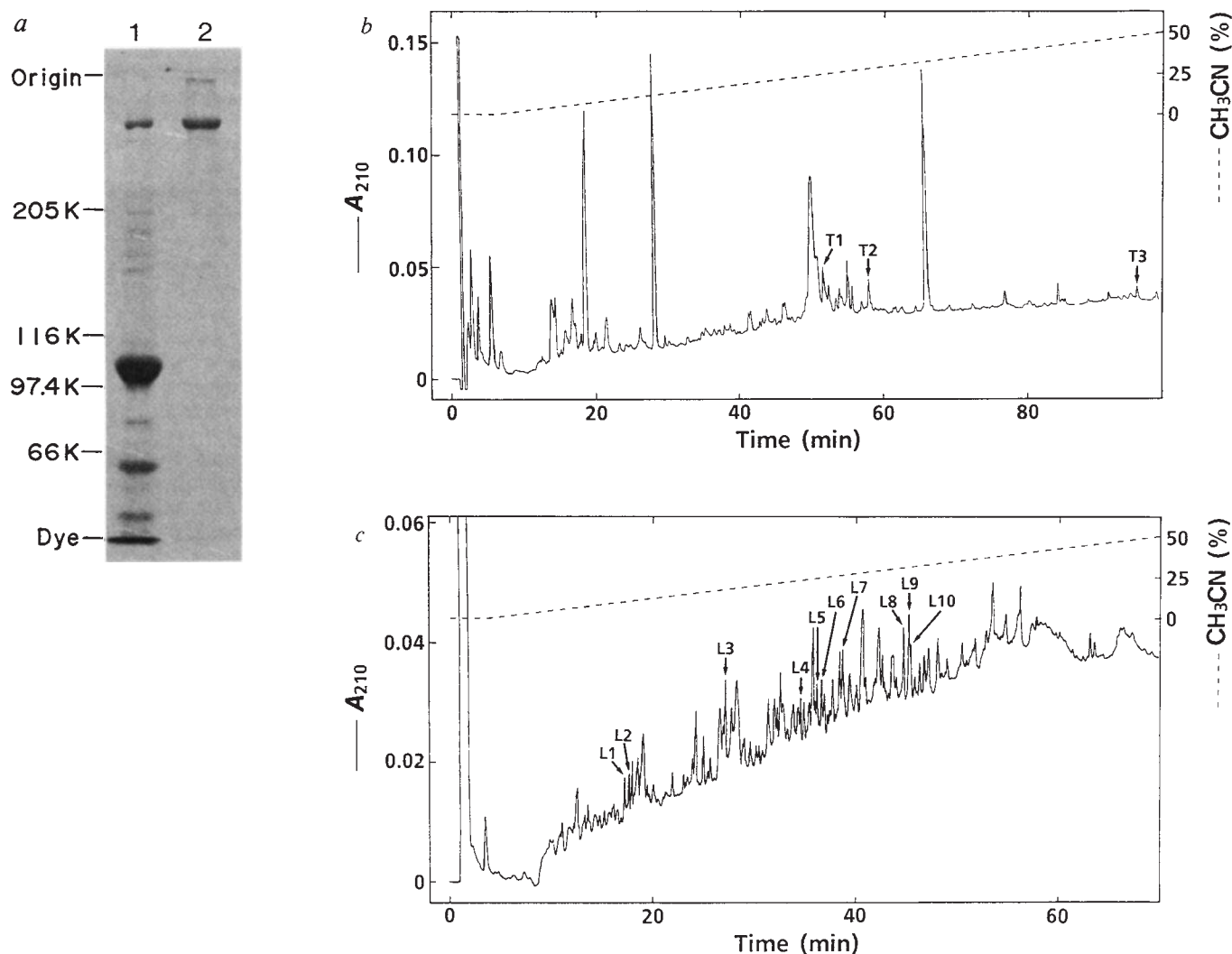


FIG. 1 Purification and partial amino-acid sequence analysis of the rabbit skeletal muscle ryanodine receptor. *a*, Coomassie brilliant-blue stain of an SDS-5% polyacrylamide gel³⁹. The samples analysed were 8 μg of the JTC fraction⁴⁰ (lane 1) and 0.5 μg of the purified ryanodine receptor (lane 2); size markers were from Sigma and bromophenol blue served as tracking dye. *b*, *c*, Reverse-phase HPLC of peptides formed from the purified ryanodine receptor by tryptic digestion (*b*) or by cyanogen bromide cleavage followed by lysyl endopeptidase digestion (*c*). Solid line and left-hand axis, absorbance at 210 nm (A_{210}); broken line and right-hand axis, acetonitrile concentration. Fraction T1 was separated by rechromatography into four fractions, two of which (T1a and T1b) were sequenced. The amino-acid sequences determined for the fractions corresponding to the absorbance peaks indicated were identical with the sequences of the following amino-acid residues deduced from the cDNA sequence (for amino-acid numbers, see Fig. 2): T1a, 2,853–

2,867; T1b, 1,241–1,258; T2, 1,865–1,891; T3, 2,416–2,426; L1, 2,826–2,837; L2, 942–951 and 4,092–4,095; L3, 1,355–1,377; L4, 2,190–2,197; L5, 468–482; L6, 2,771–2,786; L7, 404–416 and 1,241–1,259; L8, 3,544–3,562; L9, 3,090–3,105 and 3,336–3,354; L10, 1,815–1,830.

METHODS. The ryanodine receptor was solubilized from the JTC fraction⁴⁰ or the junctional face membrane (JFM) fraction⁴¹ from rabbit skeletal muscle and was purified by heparin-agarose chromatography⁴ followed by gel permeation HPLC under denaturing conditions¹⁹. After dialysis⁴², 450 μg of the purified preparation was digested with trypsin¹⁹, or 200 μg subjected to cyanogen bromide cleavage (2.5 mg in 0.1 ml of 70% formic acid) followed by lysyl endopeptidase digestion (1 μg in 0.2 ml of 10 mM Tris-HCl, pH 9.0). The digested peptides were fractionated by reverse-phase HPLC and sequenced¹⁹.

FIG. 2 (Overleaf) Nucleotide sequence of cloned cDNA encoding the rabbit skeletal muscle ryanodine receptor, together with the preceding genomic DNA sequence, and the deduced amino-acid sequence. The nucleotide sequences determined with the cloned cDNA and genomic DNA are shown in capital and small letters, respectively. Nucleotide residues are numbered in the 5' to 3' direction from the first residue of the ATG initiation triplet (the preceding residues are indicated by negative numbers). Amino-acid residues are numbered from the initiating methionine. Numbers of the nucleotide and amino-acid residues at the right-hand end of the individual lines are given. The putative mRNA start site is marked with an asterisk. Sequences of the CAAT box, GC box, CCCCAGCC motif and polyadenylation signal are underlined. Nucleotide 15,230 is followed by a poly(dA) tract. The putative transmembrane segments M1-M4 are overlined; the termini of each segment are tentatively assigned on the basis of the hydropathicity profile and the amino-acid sequence.

METHODS. Double-stranded cDNAs were synthesized⁴³ with rabbit skeletal muscle poly(A)⁺ RNA¹⁹ as template and with random hexamer, oligo(dT) or a specific oligodeoxyribonucleotide as primer, using the cDNA synthesis system (Bethesda Research Laboratories). The cDNAs synthesized with random hexamer or oligo(dT) were methylated by *EcoRI* methylase, blunted by T4 DNA polymerase and ligated with an *EcoRI* linker. After *EcoRI* digestion, cDNAs were fractionated in 1% agarose gel. Fractions longer than ~0.8 kilobase pairs (kb) were pooled and ligated into pUC18 or pBluescript KS(+) (Stratagene). The randomly primed cDNA library was screened with

the 5'-end-labelled probes 5'-TCNGG^TTCGATCAT-3' and 5'-TC^TTC^TTCNGT^AAA-3' (N denoting a mixture of A, T, G and C), synthesized on the basis of partial amino-acid sequences of peptide T2, to yield pRR72 which hybridized with both the probes. Restriction fragments from pRR72 were used as probes for screening the randomly primed cDNA library to clone cDNAs extending in both directions. Similar cloning procedures were repeated to isolate adjacent cDNAs successively. pRR705, which carries the 5'-terminal cDNA sequence, was selected from the cDNAs that were prepared with the synthetic primer 5'-CTCTCCTGTTCATCCT-3' (complementary to nucleotides 416-432), tailed with poly(dC) and cloned into poly(dG)-tailed pBR322 (ref. 19). pRR616, which carries the 3'-terminal cDNA sequence including the poly(dA) tract, was isolated from the oligo(dT)-primed cDNA library. The rabbit genomic DNA clone λRRG3, which harbours a ~15-kb sequence including the promoter region and the first exon (extending from the putative mRNA start site to nucleotide 48), was isolated by screening a Charon 4A library⁴⁴ with the 0.2-kb *PstI*(vector)/*PstI*(69) fragment from pRR705 as probe. The cDNA clones used for nucleotide sequence analysis are as follows (see Fig. 4a): pRR705 (carrying nucleotides -131 to 429), pRR256 (106-1,925), pRR229 (1,443-4,431), pRR72 (4,240-6,566), pRR203 (6,264-8,579), pRR329 (7,631-9,394), pRR308 (8,501-9,980), pRR359 (9,559-11,349), pRR451 (10,650-12,452), pRR616 (12,344-15,230 and the poly(dA) tract). DNA sequencing^{45,46} was carried out on both strands.

example, regions encompassing amino-acid residues 4,253-4,264, 4,407-4,416 and 4,489-4,499 which are assigned to the cytoplasmic side of the SR membrane and are close to segment M1. The nucleotide-binding consensus sequence GXGXXG (ref. 22) is found at several sites, including residues 4,449-4,454 or 4,452-4,457 which are located close to the EF-hand-like sequences on the cytoplasmic side. There are regions with clustered basic residues interrupted with mostly hydrophobic residues (residues 3,614-3,637 and 4,295-4,325) which might interact with calmodulin²³.

Expression of cDNA

An expression plasmid (pRRS7) was constructed which carries the entire protein-coding sequence of the ryanodine receptor cDNA, linked to the simian virus 40 (SV40) early promoter, and the neomycin-resistance marker gene (Fig. 4a, b). CHO cells were transfected with pRRS7, and G418-resistant clones that were shown by RNA blot hybridization analysis¹⁶ to produce ryanodine receptor mRNA were selected. Immunoblotting analysis of membrane preparations using monoclonal antibody against the rabbit skeletal muscle ryanodine receptor revealed that the transformed clones C7311 and C798 produced a protein indistinguishable in M_r from the ryanodine receptor (Fig. 5a). This result was confirmed by experiments with *Xenopus* oocytes injected with the mRNA that was synthesized *in vitro* by transcription of the ryanodine receptor cDNA linked to the bac-

teriophage SP6 promoter. Such oocytes, incubated with [³⁵S]methionine, yielded a radiolabelled protein that was specifically immunoprecipitated with the monoclonal antibody and was indistinguishable in M_r from the ryanodine receptor (data not shown).

Figure 5b shows that membrane preparations from the transformed CHO cells were capable of binding ryanodine; negligible ryanodine binding was found for nontransfected CHO cells. Scatchard analysis yields an apparent dissociation constant (K_D) of 19 nM, which is in the same range as the K_D measured with the junctional terminal cisternae (JTC) fraction of rabbit skeletal muscle (15-27 nM).

Discussion

Computer survey detects no extensive amino-acid sequence similarity between the ryanodine receptor and other channels of known sequence (for example, refs 19, 24-29). However, the four putative transmembrane segments located in the carboxy-terminal region are reminiscent of those of the subunits of the nicotinic acetylcholine receptor²⁴ (nAChR) and related receptors^{25,26}. The region encompassing segments M2 and M3 of the ryanodine receptor shows some sequence similarity to the M2-M3 region of the nAChR subunits, as exemplified in Fig. 6a for the α -subunit; note that the assigned transmembrane orientation of segments M2 and M3 of the ryanodine receptor is opposite to that of the nAChR subunits²⁴. Electron-micro-

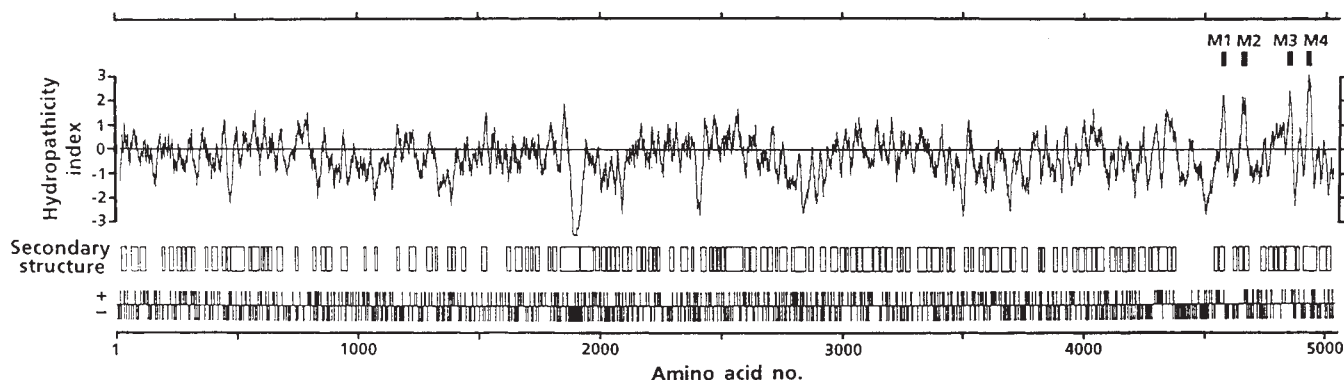


FIG. 3 Hydropathicity profile and predicted secondary structures of the ryanodine receptor. The averaged hydropathicity index²⁷ of a nonadecapeptide composed of amino-acid residues $i-9$ to $i+9$ is plotted against i , the amino-acid number. The positions of the predicted structures of α -helix and/or β -sheet¹⁸ that have a length of 10 or more residues are shown by

open boxes. The positions of the positively charged residues (Lys and Arg) and negatively charged residues (Asp and Glu) are indicated by upward (+) and downward (-) vertical lines, respectively. The positions of the putative transmembrane segments M1-M4 are indicated by filled boxes.

[illegible]

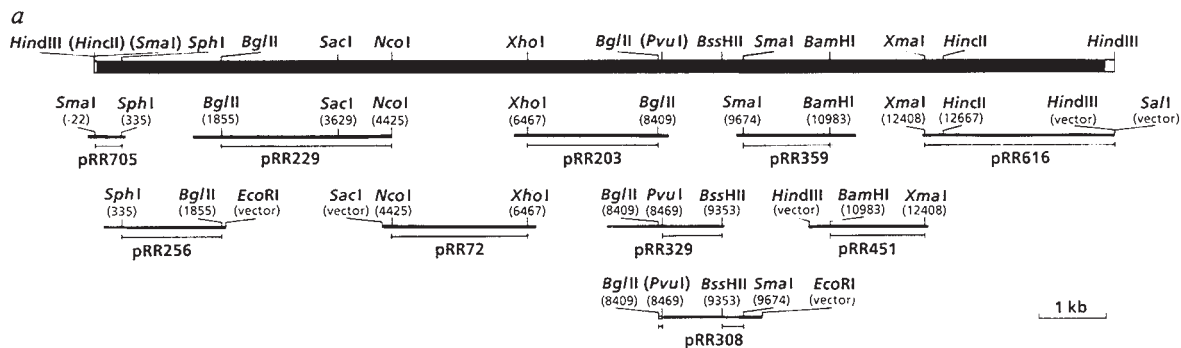
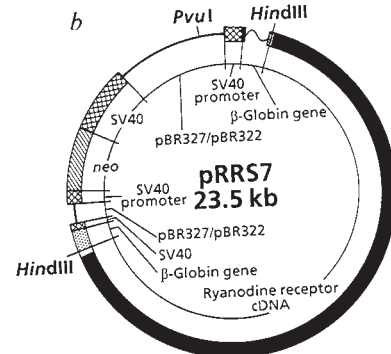


FIG. 4 Expression plasmid pRRS7 carrying the rabbit skeletal muscle ryanodine receptor cDNA. *a*, Restriction endonuclease sites used for construction of pRRS7. Endonuclease sites that no longer exist in pRRS7 are in parentheses. The protein-coding region is represented by a closed box, and the 5'- and 3'-noncoding regions by open boxes. The cDNA clones used are shown by thick lines (the open area indicating synthetic DNA), and their fragments constituting pRRS7 by bars underneath. A scale of 1 kb is given. *b*, Schematic representation of pRRS7. The origin of each segment is shown on the inner circle. The ryanodine receptor cDNA is indicated as in *a*. The SV40 sequences are shown by cross-hatched boxes; the orientation of the early promoter is such that transcription occurs clockwise. The rabbit β -globin gene sequences are indicated by stippled boxes, and its intronic sequence by a wavy line. The neo^r marker gene is represented by a hatched box, and the sequences of the plasmids pBR327 and pBR322 by lines. The HindIII sites connecting the ryanodine receptor cDNA with the pKNH vector⁴⁷ and the PvuII site at which pRRS7 was linearized before transfection of CHO cells are shown.

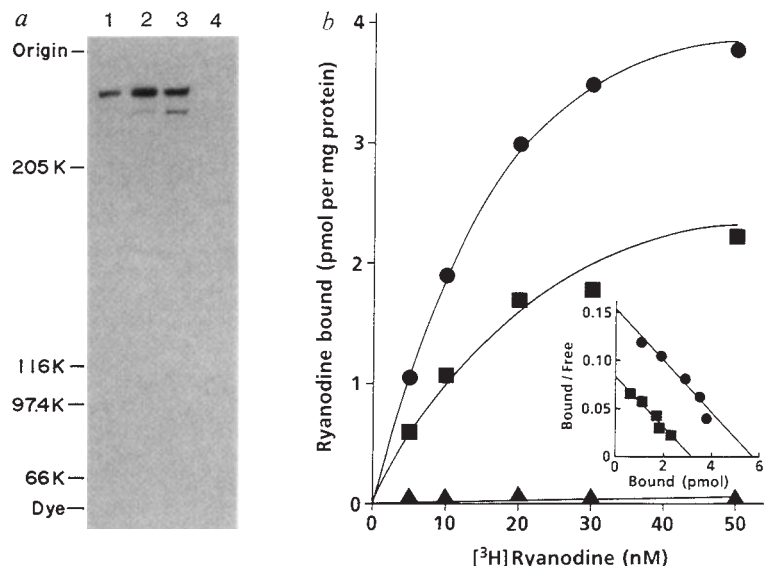
METHODS. The expression plasmid pRRS7 was constructed as follows. The SmaI(-22)/SphI(335) fragment from pRR705 and the 1.6-kb SphI(335)/EcoRI(vector) fragment from pRR256 were ligated with the EcoRI/HindIII fragment from pUC18 to yield pRRS1a; the 2-base-pair deletion from the HindIII site which occurred during the construction of pRRS1a generated an in-frame ATG triplet located 33 base pairs upstream from the translational initiation codon, but it is most unlikely that translation is initiated at the upstream ATG because it is preceded by CTTGC and is followed by C (ref. 15). The 4.8-kb SacI(vector)/NcoI(4,425) fragment containing pUC18 from pRR72 was ligated with the SacI(3,629)/NcoI(4,425) fragment from pRR229 to yield pRRS1b. The 1.9-kb HindIII(vector)/BglII(1,855) fragment from pRRS1a, the BglII(1,855)/SacI(3,629) fragment from pRR229 and the 5.6-kb HindIII(vector)/SacI(3,629) fragment containing pUC18 from pRRS1b were ligated to yield pRRS1. The XhoI(6,467)/BglII(8,409) fragment from pRR203, the BglII(8,409)/BssHII(9,353) fragment from pRR329 and the 0.63-kb BssHII(9,353)/EcoRI(vector) fragment from pRR308 were ligated with the EcoRI/XhoI fragment from pBluescript SK(-) to yield pRRS2a. The 6.1-kb BamHI(vector)/SmaI(9,674) fragment from pRRS2a was ligated



with the SmaI(9,674)/BamHI(10,983) fragment from pRR359 to yield pRRS2. For deleting the PvuII(8,469) site from pRRS2, the G residue 8,466 was converted to A by replacing the BglII(8,409)/PvuII(8,469) fragment with synthetic DNA to yield pRRS2M without causing an amino-acid substitution. The 1.8-kb HindIII(vector)/XmaI(12,408) fragment from pRR451, the XmaI(12,408)/HindIII(12,667) fragment from pRRS16 and the 2.6-kb HindIII(12,667)/SalI(vector) fragment from pRR616 were ligated with the SalI/HindIII fragment from pUC19 to yield pRRS3. The 6.5-kb HindIII(vector)/XhoI(6,467) fragment from pRRS1, the XhoI(6,467)/BamHI(10,983) fragment from pRRS2M and the 4.3-kb BamHI(10,983)/XbaI(vector) fragment from pRRS3 were ligated with the XbaI/HindIII fragment from pSP64 to yield pRRS6. Finally the 15.3-kb HindIII fragment containing the entire protein-coding sequence from pRRS6 was cloned into the HindIII site of pKNH to yield pRRS7.

FIG. 5 Expression of the cloned cDNA encoding the rabbit skeletal muscle ryanodine receptor in CHO cells. *a*, Immunoblotting analysis of the JTC fraction (12 μ g protein) from rabbit skeletal muscle (lane 1) and membrane preparations (100 μ g protein) from C7311 cells (lane 2), C798 cells (lane 3) and nontransfected CHO cells (lane 4), using monoclonal antibody mAbRR2 against the rabbit skeletal muscle ryanodine receptor. *b*, Binding of [³H]ryanodine to membrane preparations from C7311 cells (●), C798 cells (■) and nontransfected CHO cells (▲); the inset shows Scatchard plots of the data.

METHODS. BALB/c mice were repeatedly immunized with the JFM fraction or purified ryanodine receptor and hybridoma cells were produced by fusion of spleen cells with X63-Ag8.6.5.3 cells⁴⁸. Hybridomas were selected using an immunodot assay to yield clone RR2. mAbRR2 (IgG1 isotype) was found to immunoprecipitate the [³H]ryanodine-labelled receptor from the solubilized JTC fraction and the protein recognized by mAbRR2 showed a mobility indistinguishable from that of the purified ryanodine receptor on SDS-5% polyacrylamide gel electrophoresis. CHO cells were transfected with PvuII-cleaved pRRS7. Clones C7311 and C798 were isolated by screening G418-resistant clones by RNA blotting analysis¹⁶ using the 15.3-kb HindIII fragment from pRRS6 as probe. For immunoblotting analysis, samples were electrophoresed on an SDS-5% polyacrylamide gel and transferred⁴⁹ to GVHP filter (Millipore). The filter was incubated with mAbRR2 (2.8 μ g ml⁻¹ of the ammonium sulphate fraction from ascites) and then with 0.5 μ Ci ml⁻¹ of ¹²⁵I-anti-mouse IgG (Amersham). After washing, the filter was subjected to autoradiography for 30 h. Ryanodine binding was assayed with membrane preparations (100 μ g protein) in a solution (0.2 ml) containing 20 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 2 mM dithiothreitol, 25 μ M CaCl₂, 1 M NaCl, 3 mM ATP and various concentrations of [³H]ryanodine (60 Ci mmol⁻¹; New England Nuclear). After incubation for



40 min at room temperature, the samples were collected on GF/C filters (Whatman) and washed with a solution containing 0.2 M choline chloride and 20 mM Tris-HCl, pH 7.4. The filters were then counted for radioactivity. The nonspecific binding (measured in the presence of 1 μ M unlabelled ryanodine) for C7311 and C798 was less than 17% of the total radioactivity bound.

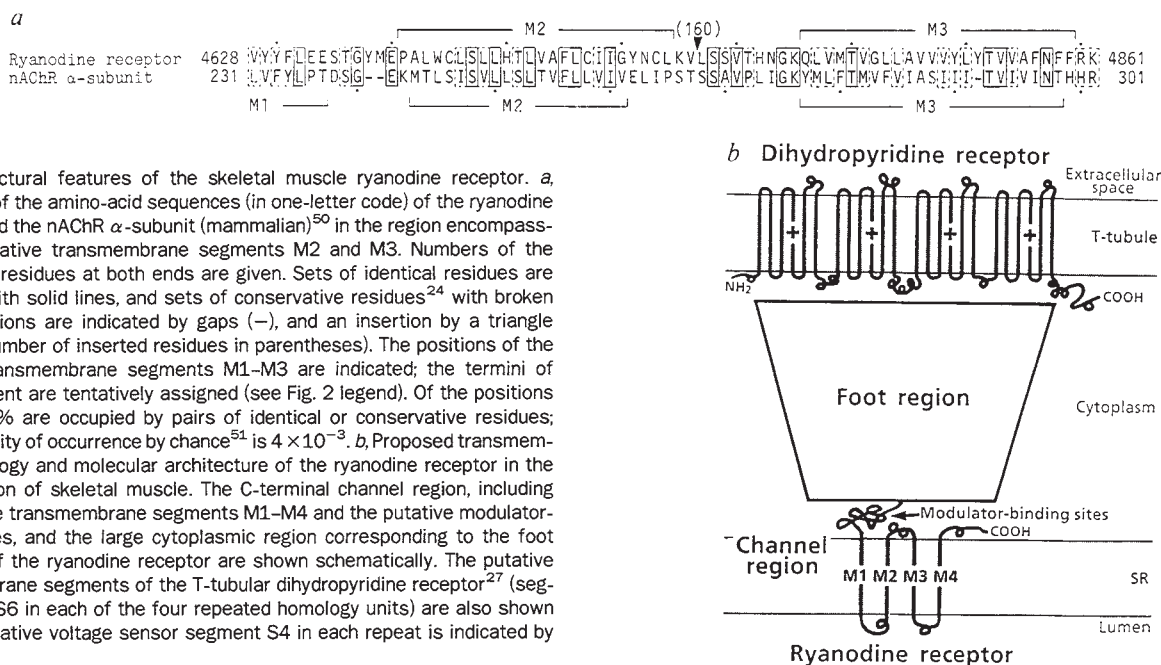


FIG. 6 Structural features of the skeletal muscle ryanodine receptor. *a*, Alignment of the amino-acid sequences (in one-letter code) of the ryanodine receptor and the nAChR α -subunit (mammalian)⁵⁰ in the region encompassing the putative transmembrane segments M2 and M3. Numbers of the amino-acid residues at both ends are given. Sets of identical residues are enclosed with solid lines, and sets of conservative residues²⁴ with broken lines. Deletions are indicated by gaps (—), and an insertion by a triangle (with the number of inserted residues in parentheses). The positions of the putative transmembrane segments M1–M3 are indicated; the termini of each segment are tentatively assigned (see Fig. 2 legend). Of the positions aligned, 49% are occupied by pairs of identical or conservative residues; the probability of occurrence by chance⁵¹ is 4×10^{-3} . *b*, Proposed transmembrane topology and molecular architecture of the ryanodine receptor in the triad junction of skeletal muscle. The C-terminal channel region, including the putative transmembrane segments M1–M4 and the putative modulator-binding sites, and the large cytoplasmic region corresponding to the foot structure of the ryanodine receptor are shown schematically. The putative transmembrane segments of the T-tubular dihydropyridine receptor²⁷ (segments S1–S6 in each of the four repeated homology units) are also shown and the putative voltage sensor segment S4 in each repeat is indicated by plus signs.

scopic and ryanodine-binding studies suggest that the ryanodine receptor forms a homo-tetrameric complex^{6,7,30}. The nAChR is known to have a heteropentameric structure with a subunit stoichiometry of $\alpha_2\beta\gamma\delta$ (ref. 31), and rings containing negatively charged residues that neighbour segment M2 of the constituent subunits have been shown to be major determinants of the rate of ion transport through the nAChR channel³². Both the calcium-release channel and the nAChR channel are permeable to monovalent and divalent cations and non-electrolytes^{30,33}. The structural and functional resemblance between the ryanodine receptor and the nAChR makes it likely that the putative transmembrane segments in the C-terminal region of the ryanodine receptor, contributed by each of the four monomeric units, surround a central pore to form the calcium-release channel. Interestingly, the cytoplasmic region preceding and close to segment M1 of the ryanodine receptor contains putative binding sites for the modulators of the calcium-release channel (see above). These observations suggest that the C-terminal portion of the ryanodine receptor, which includes both the channel-forming region and the modulator-binding sites, is responsible for the calcium release channel activity, whereas the preceding

large cytoplasmic region corresponds to the foot structure. The proposed architecture of the ryanodine receptor molecule, shown schematically in Fig. 6b, is consistent with the electron-microscopic observations reported^{6,7,14}. There is evidence that the dihydropyridine receptor in the T-tubule membrane of skeletal muscle functions not only as the slow calcium channel but also as an essential component of E–C coupling, probably as the voltage sensor^{27,34,35}. Recent morphological studies suggest a direct interaction between the ryanodine receptor and a protein component in the junctional T-tubule membrane³⁶. Thus it is an attractive hypothesis that the large cytoplasmic region of the ryanodine receptor directly interacts with the cytoplasmic region of the dihydropyridine receptor to effect E–C coupling.

Another way of releasing Ca^{2+} from an intracellular store involves the inositol 1,4,5-trisphosphate (InsP_3)-gated calcium channel, which is present in a variety of cells³⁷. The InsP_3 receptor with an M_r of 260K has been purified³⁸. It is conceivable that this channel is structurally related to the ryanodine receptor.

Sequence data from this paper will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession number X15209. □

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- Endo, M. *Physiol. Rev.* **57**, 71–108 (1977).
- Martonosi, A. N. *Physiol. Rev.* **64**, 1240–1320 (1984).
- Franzini-Armstrong, C. *J. Cell Biol.* **47**, 488–499 (1970).
- Inui, M., Saito, A. & Fleischer, S. *J. Biol. Chem.* **262**, 1740–1747 (1987).
- Imagawa, T., Smith, J. S., Coronado, R. & Campbell, K. P. *J. Biol. Chem.* **262**, 16636–16643 (1987).
- Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q.-Y. & Meissner, G. *Nature* **331**, 315–319 (1988).
- Saito, A., Inui, M., Radermacher, M., Frank, J. & Fleischer, S. *J. Cell Biol.* **107**, 211–219 (1988).
- Hymel, L., Inui, M., Fleischer, S. & Schindler, H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 441–445 (1988).
- Berk, A. J. & Sharp, P. A. *Cell* **12**, 721–732 (1977).
- Breathnach, R. & Chambon, P. *Rev. Biochem. Sci.* **50**, 349–383 (1981).
- Kadonaga, J. T., Jones, K. A. & Tjian, R. *Trends biochem. Sci.* **11**, 20–23, (1986).
- Korczak, B. et al. *J. Biol. Chem.* **263**, 4813–4819 (1988).
- Ellis, S. B. et al. *Science* **241**, 1661–1664 (1988).
- Wagenknecht, T. et al. *Nature* **338**, 167–170 (1989).
- Kozak, M. *Nucleic Acids Res.* **12**, 857–872 (1984).
- Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201–5205 (1980).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
- Chou, P. Y. & Fasman, G. D. *Rev. Biochem.* **47**, 251–276 (1978).
- Noda, M. et al. *Nature* **312**, 121–127 (1984).
- Meissner, G. *Biochemistry* **25**, 244–251 (1986).
- Kretsinger, R. H. *Rev. Biochem.* **45**, 239–266 (1976).
- Wierenga, R. K. & Hol, W. G. J. *Nature* **302**, 842–844 (1983).
- Blumenthal, D. K. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3187–3191 (1985).
- Numa, S. *Chemia Scripta* **26B**, 173–178 (1986).
- Grenningloh, G. et al. *Nature* **328**, 215–220 (1987).
- Schofield, P. R. et al. *Nature* **328**, 221–227 (1987).
- Tanabe, T. et al. *Nature* **328**, 313–318 (1987).
- Tempel, B. L., Papazian, D. M., Schwarz, T. L., Jan, Y. N. & Jan, L. Y. *Science* **237**, 770–775 (1987).
- Paul, D. L. *J. Cell Biol.* **103**, 123–134 (1986).

- Smith, J. S. et al. *J. gen. Physiol.* **92**, 1–26 (1988).
- Brisson, A. & Unwin, P. N. T. *Nature* **315**, 474–477 (1985).
- Imoto, K. et al. *Nature* **335**, 645–648 (1988).
- Hille, B. *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, Massachusetts, 1984).
- Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. *Nature* **336**, 134–139 (1988).
- Rios, E. & Brum, G. *Nature* **325**, 717–720 (1987).
- Block, B. A., Imagawa, T., Campbell, K. P. & Franzini-Armstrong, C. *J. Cell Biol.* **107**, 2587–2600 (1988).
- Berridge, M. J. *Rev. Biochem.* **56**, 159–193 (1987).
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. *J. Biol. Chem.* **263**, 1530–1534 (1988).
- Laemmli, U. K. *Nature* **227**, 680–685 (1970).
- Saito, A., Seiler, S., Chu, A. & Fleischer, S. *J. Cell Biol.* **99**, 875–885 (1984).
- Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A. & Fleischer, S. *J. Cell Biol.* **103**, 741–753 (1986).
- Kawakami, K. et al. *Nature* **316**, 733–736 (1985).
- Gubler, U. & Hoffman, B. J. *Gene* **25**, 263–269 (1983).
- Maniatis, T. et al. *Cell* **15**, 687–701 (1978).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
- Fukuda, K. et al. *Nature* **335**, 355–358 (1988).
- Köhler, G. & Milstein, C. *Nature* **256**, 495–497 (1975).
- Towbin, H., Staehelin, T. & Gordon, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4354 (1979).
- Noda, M. et al. *Nature* **305**, 818–823 (1983).
- Toh, H., Hayashida, H. & Miyata, T. *Nature* **305**, 827–829 (1983).

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Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells

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Genes expressed in erythroid cells contain binding sites for a cell-specific factor believed to be an important regulator for this haematopoietic lineage. Using high-level transient expression in mammalian cells, we have identified complementary DNA encoding the murine protein. The factor, a new member of the zinc-finger family of DNA-binding proteins, is restricted to erythroid cells at the level of RNA expression and is closely homologous between mouse and man.

CELLULAR commitment and differentiation depend on the establishment of specific patterns of gene expression achieved through networks of regulatory factors. Many of these factors bind in a sequence-specific manner to promoter or enhancer elements of expressed genes¹. Others interact with DNA-binding proteins to modulate transcription. Cell-specific gene expression appears to reflect the interplay of multiple ubiquitous factors with a more limited array of tissue-restricted factors².

The regulation of the globin gene family in the erythroid lineage of haematopoietic cells provides an excellent system in which to study tissue and developmental stage-specific gene expression³. In all species, switches in the expression of specific globin chains occur during both ontogeny and cell differentiation. Transfection of cultured lines and experiments with transgenic mice have identified cell-specific *cis*-acting DNA sequences both 5' and 3' to the transcription initiation sites of globin genes⁴⁻⁸. Moreover, a dominant control region (DCR) upstream of the embryonic human ϵ -globin gene⁹ confers high-level erythroid-specific expression on globin or heterologous sequences^{10,11}.

Factors that bind *in vitro* to promoter, intron and enhancer sequences of globin genes have been identified in chicken, mouse and human erythroid cells¹²⁻²⁰. Although the majority of these activities are found in a wide variety of cell types, an erythroid-specific activity recognizing a core DNA sequence of the general form {(A/T)GATA(A/G)}^{14,21} has been observed in analyses of chicken globin genes²¹⁻²³, the human β - and γ -globin genes^{15,17,20,24,25}, the human 3' β -enhancer¹⁴, and the mouse $\alpha 1$ - and β -major genes^{16,19,26}. Binding sites are also present in the A γ -globin enhancer and hypersensitive site II within the DCR (our unpublished data) and in the erythroid-specific promoter of the porphobilinogen deaminase gene^{19,26}. The activity of the factor does not appear to be restricted to a particular developmental stage of erythroid cells^{14,20,21}. The factor has been termed Eryf 1 (ref. 21), NF-E1 (ref. 14), EF-1 (ref. 26), EF γ a (ref. 17), or GF-1 (ref. 20). Herein, the activity will be referred to as GF-1.

Indirect evidence for transcriptional activation by this factor

has been reported. First, base substitutions in the chicken 3' β -enhancer that disrupt one or both of two binding motifs reduce or abolish activity of chimaeric constructs used to transfect primary erythroblasts^{21,27}. Second, increased activity of the human A γ -globin gene promoter bearing a T $\rightarrow$ C mutation at position -175 is mediated by the factor²⁰. Moreover, deletion analyses of the human 3' β -enhancer and the mouse $\alpha 1$ -globin promoter^{4,8,14} have correlated functional regions and GF-1 binding sites.

The presence of binding sites within nearly all characterized genes expressed specifically in erythroid cells, the stage-independent nature of the activity in these cells, and data relating altered enhancer or promoter function to mutations of the DNA-binding site all strongly suggest that GF-1 is a major regulatory protein of the erythroid lineage. To characterize this factor and begin to address its regulation and role in erythroid gene expression, we have isolated cDNA encoding the factor by transient expression in mammalian cells. This novel strategy is both rapid and simple, and should prove generally useful in the cloning of other cell-specific or inducible DNA-binding proteins. GF-1 messenger RNA is expressed specifically in erythroid cells and codes for a protein containing two very similar Zn-finger domains.

Results

Characterization of GF-1. GF-1 activity has been detected in nuclear extracts of erythroid cells of chicken, mouse and human origin^{14-17,19-21,24-26}. We have focused on the human and murine activities. We first established whether the activity of GF-1 was associated with a single polypeptide chain or was heterodimeric. Conventional and affinity chromatography²⁸ of nuclear proteins of human erythroleukaemia cells (K562) enriched for polypeptides of relative molecular mass (M_r) 115, 50, 38 and 20K (Fig. 1a, lanes 2-4). The presence of protease inhibitors throughout purification reduced the relative contribution of the 38 and 20K species (lane 5).

To correlate polypeptides with specific DNA-binding activity, each was eluted from SDS-polyacrylamide gels, denatured, re-natured and then used in a gel retardation assay^{29,30}. Activity was associated with the 50, 38 and 20K polypeptides (Fig. 1b, lanes 4-6). As evident in Fig. 1b (lanes 1, 2), the 38 and 20K polypeptides arise during purification. We conclude that GF-1 DNA-binding activity is associated with a single-chain 50K polypeptide and that proteolytic fragments of 38 and 20K retain DNA-binding capacity.

Although proteolytic fragments retain DNA-binding activity, they produce different DNase I footprints on the human A γ -globin promoter (Fig. 1c). The two core motifs protected by GF-1 in this promoter are both strongly protected by unproteolysed GF-1 (lane 4). Previously we have shown that this extended footprint results from the binding of a single GF-1 molecule,

rather than two molecules each binding a single motif²⁰. The 38K proteolytic fragment protects the proximal motif less completely (lanes 5); the 20K fragment protects only the distal motif (lane 6).

When intact GF-1 is used in gel retardation assays under conditions in which DNA probe is limited, we observe an additional complex that migrates more slowly than that containing a single bound molecule (Fig. 1*b*, lane 4 and Fig. 3*a*, below). Because the slower complex is also seen when a mouse $\alpha 1$ -globin promoter fragment containing only one binding site is used (data not shown), we infer that this additional complex reflects either a dimer of GF-1 bound to a single DNA molecule or a dimer of protein-DNA complexes.

Cloning of murine GF-1 cDNA by expression in mammalian cells. The assignment of GF-1 activity to a single polypeptide chain raised the possibility that expression of recombinant clones in non-erythroid cells might permit identification of cDNAs encoding the factor. We reasoned that high-level transient expression might allow detection of the characteristic DNA-binding activity through gel retardation assay of mammalian cells transfected with pools of cDNAs (Fig. 2*a*). Pilot experiments indicated that crude lysates of cultured K562 or mouse erythroleukaemia (MEL) cells retained GF-1 activity and that only 2,000 cells were required in a mixture of negative cells to generate a shifted band.

To identify GF-1 cDNA we used a cDNA library of MEL cells which was constructed in the expression vector pXM and contained $\sim 2 \times 10^5$ independent clones³¹. Among 200 pools of $\sim 1,000$ –2,000 cDNA clones used to transfect monkey kidney COS-1 cells, two (numbers 127 and 13) reproducibly gave complexes (Fig. 2*b*) that were specifically competed with excess non-radioactive DNA. Cells transfected with pool number 127 yielded a gel-shift band (solid arrow) comigrating with the authentic GF-1 complex of either mouse or human origin. DNA of pool number 13 yielded a complex of faster mobility (open arrow). In three successive rounds of sib selection during which progressive enrichment was observed (Fig. 2*c*) two isolates of the single positive cDNA in pool number 127 were obtained. Subsequent hybridization to bacterial colonies of pool number 13 permitted recovery of clone 13.

Sequence-specific DNA-binding in gel-shift assays, DNase I footprinting and direct protein sequencing of human GF-1 provide definitive evidence that the cDNA derived from pool number 127 encodes authentic murine GF-1. Nuclear extract of COS cells transfected with pool number 127 cDNA contains gel-shift activity with specificity of DNA binding identical to that of purified 50K polypeptide (Fig. 3*a*). Oligonucleotides containing mutations in GF-1 binding sites in the γ -globin promoter and γ -globin 3' enhancer compete gel-shift complex formation less efficiently than wild-type sequences. The extract of transfected

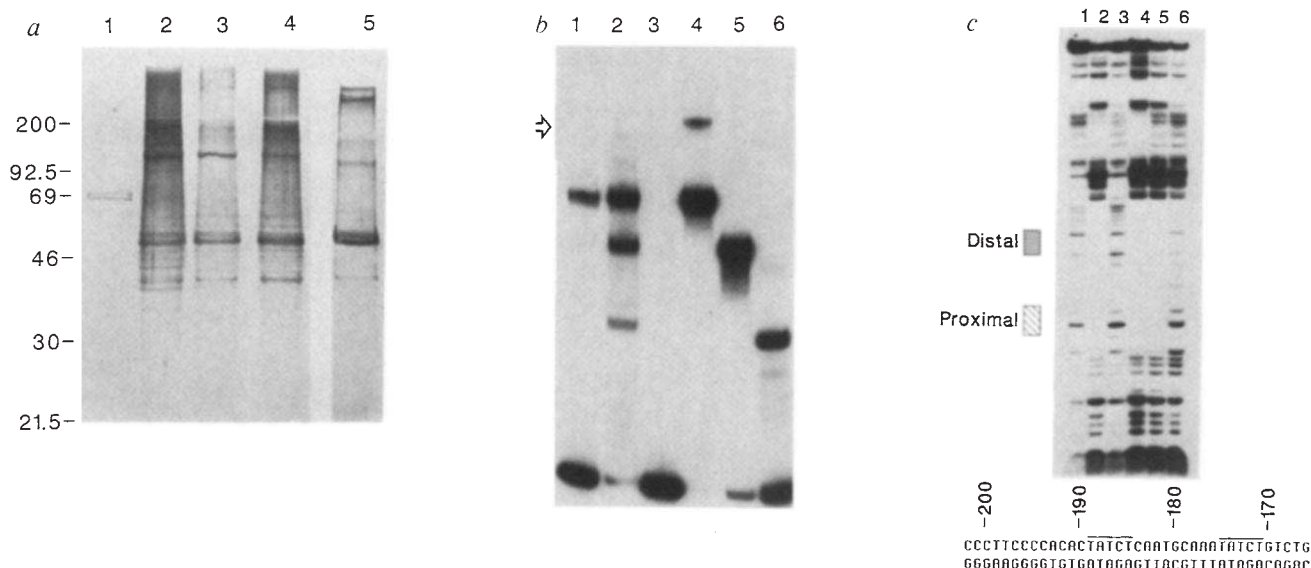


FIG. 1 Characterization of purified human GF-1. *a*, Affinity-purified GF-1. K562 nuclear protein, initially fractionated on heparin-sepharose, was purified by passage through a DNA-affinity column bearing a binding site from the mouse $\alpha 1$ -globin promoter. Lane 1, bovine serum albumin standard (200 ng); (lanes 2–4), polypeptides eluted after one, two or three passes, respectively; lane 5, polypeptides eluted after three passages through the affinity column in the presence of protease inhibitors. *b*, Gel retardation analysis of purified GF-1. Lane 1, total K562 extract; lane 2, affinity-purified GF-1; (lanes 3–6, 115, 50, 38 and 20K polypeptides eluted from SDS-polyacrylamide gel, denatured and renatured. The putative dimer-complex is indicated by the open arrow. *c*, DNase I footprinting of purified GF-1 polypeptides. Lane 1, no added protein; lane 2, affinity-purified GF-1; lanes 3–6, 115, 50, 38 and 20K eluted polypeptides. The distal and proximal TATCT motifs are denoted. An additional footprint, present in the -215 region in the vicinity of a low-affinity binding motif²⁰, is seen more distally.

METHODS. Gel-retardation and DNase I footprinting assays were performed as previously described²⁰. A 117 base pair (bp) *Hinf*I–*Nco*I fragment derived from the human γ -globin promoter was radiolabelled and used in both assays. Purification of GF-1: nuclear extracts from K562 cells were prepared as previously described²⁰. Approximately 80 ml (5 mg ml^{-1} protein) of nuclear extract in buffer D (ref. 66) was applied to heparin-sepharose. The column was washed with buffer D before step elution by 0.1-M increments of KCl in buffer D. The presence of binding activity for Oct-1 (ref. 45) and GF-1 was assayed by gel retardation. Oct-1 and GF-1 eluted at 0.3 and

0.5 M KCl, respectively. Pooled GF-1 activity was adjusted to 55% ammonium sulphate, and the precipitated protein was resuspended and dialysed in buffer Z (ref. 67). For affinity purification, the sequence GATCTCCGGCAACT-GATAAGGATTCCTG was concatamerized and coupled to Sepharose CL-2B (ref. 28). The dialysed GF-1 sample was mixed with poly (dl.dC) ($20 \mu\text{g ml}^{-1}$) and incubated at 4°C for 30 min, and then applied at 15 ml h^{-1} to three DNA-affinity columns of 1-ml bed volume each. The retained GF-1 activity was eluted with 2-ml steps of buffer Z containing 0.1-M increments of KCl. Each fraction was analysed for binding activity by gel-shift assay and for protein content by SDS-PAGE, as visualized by silver staining. For further purification, GF-1-containing fractions, eluted at 0.3–0.5 M KCl, were collected, adjusted to 0.1 M KCl in buffer Z, incubated with $5 \mu\text{g ml}^{-1}$ poly (dl.dC), applied to a 1-ml column, and eluted as above. Renaturation of GF-1 binding activity. The method of Briggs *et al.*⁶⁷ was used with slight modification. Approximately $1 \mu\text{g}$ of affinity-purified sample containing all four major species was separated by SDS-PAGE. The regions of the gel containing the 115, 50, 38 and 20K polypeptides were localized by parallel lanes stained with silver reagents, excised and incubated in 1 ml elution buffer⁶⁷. The eluted protein was concentrated by acetone precipitation and resuspended in $50 \mu\text{l}$ of denaturation buffer. After incubation at 25°C for 4 h, the denatured protein was renatured at 4°C by dialysis. Gel-shift and DNase I footprinting assays were performed with the eluted samples in the absence of nonspecific competitor (poly (dl.dC)).

COS cells also yields a DNase I footprint on the $\text{A}\gamma$ -globin promoter that is indistinguishable from that obtained with the 50K GF-1 polypeptide purified from K562 cells (Fig. 3b). Finally, as indicated below, peptide sequencing of human GF-1 provided amino-acid sequences that matched the predicted murine protein.

Murine GF-1 contains two Cys-Cys zinc fingers. The nucleotide sequence of full-length GF-1 cDNA and its deduced protein sequence are displayed in Fig. 4a. The cDNA predicts a polypeptide of 413 amino acids (calculated M_r , 42.6K). The assignment of the initiation codon to the first ATG in the sequence is supported by our findings with cDNA number 13. The 5' extent of this cDNA, which encodes a polypeptide yielding a faster mobility gel retardation complex (Fig. 2b), is at nucleotide 271. We infer that a methionine codon downstream of this position (at nucleotides 318–320) is used to translate the truncated polypeptide. Hence, polypeptide synthesis from cDNA clone 127 must initiate upstream at position 69–71.

GF-1 is not overtly similar to other proteins or to other recently sequenced DNA-binding proteins. The predicted sequence is rich in serine, threonine and proline (12%, 8%, 9.4%, respectively). An acidic domain, characteristic of some transcriptional activators³², is not evident. The somewhat unusual amino-acid content, similar to that of other transcription factors^{33,34}, probably accounts for the small discrepancy between the calculated and observed sizes of the protein³³, although post-translation modifications such as phosphorylation or *O*-glycosylation³⁵ might contribute as well.

Inspection of the predicted polypeptide reveals two domains that are very similar to each other (Fig. 4b). The pattern Cys-X-Asn-Cys-X₁₇-Cys-Asn-X-Cys is repeated twice. This arrangement is most reminiscent of Zn finger proteins of the C_x class^{36,37}.

Within the repeats strong conservation is found in the central area between the cysteine pairs (Leu-Trp-Arg-Arg), at the base of the carboxyl cysteine pair (Gly-Leu-Tyr-X-Lys), and downstream of the base of the finger (Asn-Arg-Pro-Leu). Two features of the putative Zn fingers are particularly noteworthy. First, within the cysteine pairs is an alternating arrangement of asparagine (Asn) residues. Second, the spacing between the cysteine pairs (17 amino acids), as well as between the putative fingers, is larger than that reported for other members of the C_x class.

Restricted expression of murine GF-1 mRNA. The presence of GF-1 activity only in erythroid cells might be due either to cell-specific expression of its mRNA or to inhibition (or inactivation) of GF-1 protein in non-erythroid cells. To distinguish between these possibilities we examined RNAs of murine cell lines and tissues by northern blot analysis (Fig. 5a). GF-1 mRNA was detected only in erythroleukaemic cells (MEL). Other haematopoietic cell lines derived from different lineages (B cells, T cells and macrophages) and primary, non-erythroid tissues did not contain detectable GF-1 mRNA. Although dimethylsulphoxide-induced maturation of MEL *in vitro* is associated with greatly increased expression of globin mRNAs, the abundance of GF-1 mRNA was only slightly increased in induced cells. We conclude that GF-1 is restricted to the erythroid lineage at the level of RNA expression.

Conservation of human GF-1 mRNA expression and protein. As the gel-shift mobility and specificity of GF-1-DNA complexes of mouse and human origin are virtually indistinguishable, we anticipated that murine and human GF-1 polypeptides might be closely related. We first examined potential cross-hybridization of the murine cDNA with human RNA. Murine GF-1 cDNA detects a 1.7-kilobase (kb) transcript in human erythro-

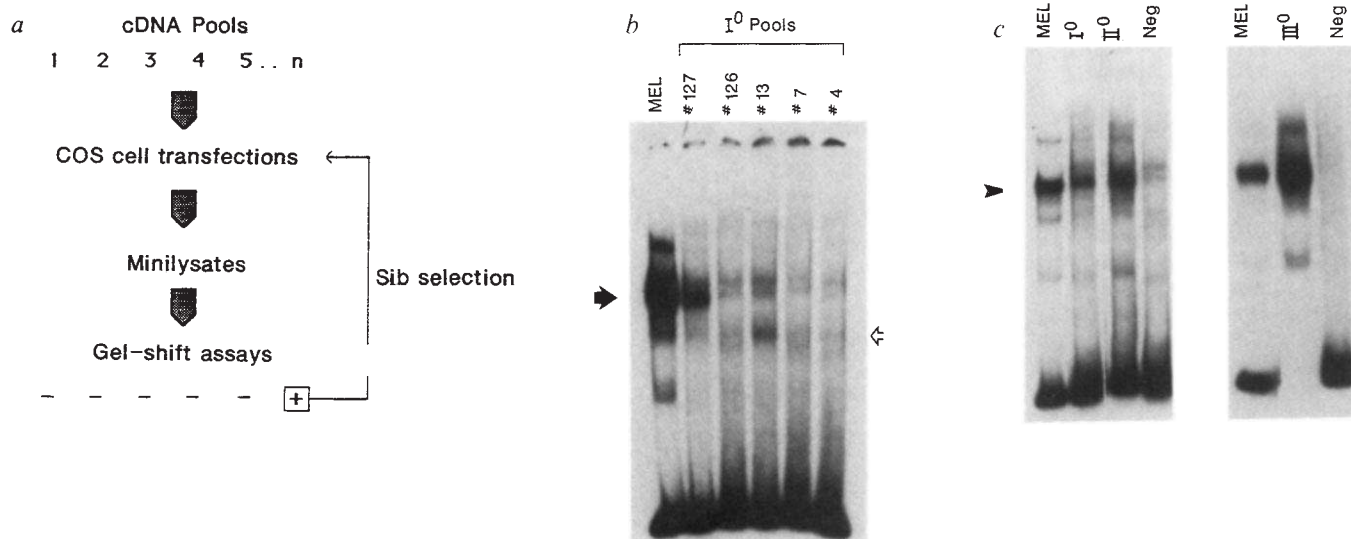


FIG. 2 Expression cloning of murine GF-1 cDNA. *a*, Scheme for cloning of GF-1 cDNA. Miniprep DNA from independent pools of HB101 bacterial transformants of the MEL-pXM cDNA library³¹ are used to transfect COS cells in individual dishes. Minilysates are prepared and examined by gel-shift assay. Pools which yield GF-1 activity in the gel-shift assay (indicated by +) are subjected to sib selection to obtain the appropriate cDNA clone. *b*, Gel-shift assay of COS-1 cells transfected with primary cDNA pools. MEL: mouse erythroleukaemia nuclear extract. Closed arrow: gel-shift complex formed by intact (50K) GF-1 bound to the *Hinfl*-*NcoI* $\text{A}\gamma$ -globin fragment. Open arrow: position of the faster-migrating complex seen with extract of COS cells transfected with pool number 13. *c*, Sib selection of GF-1 cDNA clone in pool number 127. I°, I°, and III° indicate assays of the primary pool transfected in COS cells, and the first and second sib-selection steps, respectively.

METHODS. Miniprep DNAs from individual pools of 1,000–2,000 MEL-cDNA recombinants were used to transfect COS-1 cells essentially as described by D'Andrea *et al.*³¹. 48–72 h after transfection, plates were rinsed with

saline and collected by centrifugation. Cell pellets were resuspended in 25 μl lysis buffer (20 mM HEPES, pH 7.9, 140 mM NaCl, 1.5 mM MgCl_2 , 0.5% NP-40) supplemented with protease inhibitors²⁰, transferred to a 1.5-ml tube, and centrifuged at 2,000 r.p.m. for 10 min. The supernatant was recovered for gel-shift assay. For gel-retardation assay, 5 μl of minilysate was incubated in a total volume of 50 μl with 2.5×10^4 c.p.m. of radiolabelled probe, 2.5 μg poly(dI.dC) for 15 min. A mutant human $\text{A}\gamma$ -globin promoter *Hinfl*-*NcoI* fragment with a T-C substitution at position -175 was used instead of the wild-type probe to reduce complex formation with Oct-1 (ref. 20). Sib selections. The primary filter of a positive pool (number 127) was divided into 72 sections. Cultures from each section were assayed by construction of a 9×8 matrix^{31,38}. In a second round of sib selection, 144 sections were assayed. In the final stage of selection, 1,000 individually picked colonies were assayed seven at a time in a 12×12 matrix to minimize a competitive growth disadvantage of clone number 127 amidst other colonies.

leukaemic cells (K562 or HEL) that is absent in other haematopoietic cell lines (U937 or MOLT 4) (Fig. 5b) and other non-erythroid lines (HeLa and HepG2, not shown). Poly(A)⁺ RNA of K562 cells is enriched for this species (open arrow). The relatively weak signal detected by cross-hybridization could be due to several factors: lower abundance of

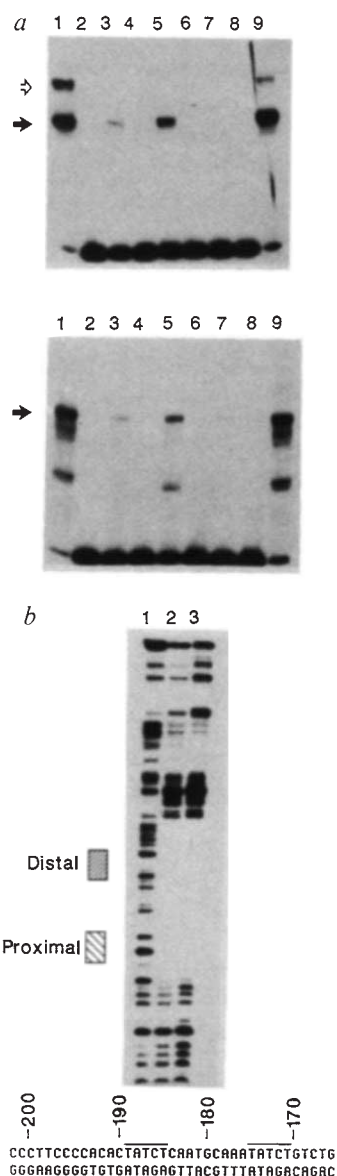


FIG. 3 Sequence-specific binding of murine GF-1 cDNA expressed in COS-1 cells. *a*, Specificity determined by gel-shift assay. Top, 50K affinity-purified GF-1 from K562 cells; bottom, nuclear extract of COS-1 cells transfected with GF-1 cDNA (clone number 127). Probe: *Hinf*I-*Nco*I fragment of the -175 T→C A γ -globin promoter. Lane 1, no competitor DNA. Lanes 2-9, unlabelled double-stranded oligonucleotides were added at 100-fold excess. Lane 2, homologous A γ -globin promoter sequences from -193 to -169; lane 3, A γ -globin DNA with C-A substitutions at -186 and -172, which reduce binding of GF-1 (ref. 20); lane 4, A γ 3' enhancer sequence AAT-TATTTTCCTTATCAGAAGCAGAGA; lane 5, corresponding G γ 3'-sequence AATTATTTTCCTCATCAGAAGCAGAGA; lane 6, human 3' β -enhancer sequence TGCTGGCTCCCTTATCATGTCCCTTATGGTGC; lane 7, chicken 3' β -enhancer En4 sequence²¹; lane 8, mouse α 1-globin promoter sequence GATCTCCGGCAACTGATAAGGATCCCTG; lane 9, heterologous DNA (-140 to -101 of the A γ -globin promoter). The major GF-1-DNA complex is indicated by the solid arrows. The complex reflecting GF-1 dimers is indicated by the open arrow. *b*, Specificity determined by DNase I footprinting assay. Probe: *Hinf*I-*Nco*I fragment of the wild-type A γ -globin promoter. Lane 1, no added protein; lane 2, extract from COS-1 cells transfected with GF-1 cDNA clone number 127; lane 3, 50K GF-1 polypeptide.

GF-1 mRNA in K562 and HEL cells; conservation of murine and human protein sequences only within a limited region, perhaps restricted to the DNA-binding domain; or broad nucleotide sequence divergence but strong conservation of coding capacity. As the level of GF-1 DNA-binding activity in nuclear extracts of MEL and K562 cells does not appear to be vastly different, we suspect that the latter two possibilities are more likely.

Subsequent to the cloning of murine GF-1 cDNA, sufficient affinity-purified human GF-1 polypeptide was prepared for enzymatic digestion and peptide sequencing. The directly determined amino-acid sequence of one peptide precisely matches a region of the predicted mouse sequence to the carboxyl side of the second putative Zn finger domain (Fig. 4b). These data provide definitive evidence that (1) the murine cDNA cloned through expression in mammalian cells encodes GF-1 protein and (2) the murine and human homologues of GF-1 are very closely related at the level of the amino-acid sequence, at least in a region flanking the putative DNA-binding domain, in spite of seemingly weak cross-hybridization of nucleotide sequences.

Discussion

A new strategy for the cloning of DNA-binding proteins. We have used high-level, transient expression in mammalian cells to identify cDNA encoding a eukaryotic DNA-binding protein. Although mammalian cell expression has been used previously to isolate cDNAs for growth factors, cell surface antigens and growth-factor receptors^{31,38-41}, to our knowledge this is the first cDNA for a transcriptional factor to be isolated by such an approach.

Several strengths of this approach deserve comment. First, the strategy should be generally applicable to the isolation of clones encoding single-chain DNA-binding proteins that are either cell-type specific or induced *de novo* in cells under specified conditions. Second, the method permits rapid comparison of expressed protein (even in primary pools) with native factor with respect to the mobility of complexes in gel-shift assay and specificity of DNA binding. This feature is particularly attractive and is to be contrasted with screening of bacteriophage λ gt11 libraries with a cognate DNA-binding site^{42,43}. In the latter instance, although cDNAs encoding DNA sequence-specific binding proteins have been identified^{42,44-46}, it is often uncertain whether they are derived from the desired protein, particularly when distinct proteins interact with similar DNA-binding sites⁴². Third, as exemplified by the identification of clone number 13 bearing a truncated cDNA, the requirement for expression of a full-length cDNA is not absolute. Fourth, the approach is rapid and sensitive.

Previously, two other methods, conventional protein purification^{28,33} and expression cloning of DNA-binding domains in the bacteriophage vector λ gt11^{42,43}, have been used to identify cDNAs encoding transcriptional factors. These methods, although often successful, have limitations. Protein purification for microsequencing or antibody production generally requires large amounts of nuclear extract, which may constitute a severe restriction when a factor is present within a minor cell type in a tissue or available only from cultured cells. Also, it is not certain that the λ gt11 cloning method will be applicable to the vast majority of DNA-binding proteins. The avidity of specific DNA-binding domains, their capacity to function as part of a fusion to β -galactosidase, and their resistance to binding to nitrocellulose, denaturation or extensive washing are critical determinants. The method we have described avoids these limitations and should complement other existing procedures.

GF-1: an erythroid-specific Zn finger DNA-binding protein. Few mammalian cell-type-specific transcription factors have been characterized thus far by direct protein and/or cDNA analysis. In B lymphoid and pituitary cells, cell-restricted DNA-binding factors (Oct-2 and GHF-1/Pit-1), critical for the transcriptional

activation of immunoglobulin and prolactin/growth hormone genes^{44,46,47-54}, are members of an extended homoeobox family⁵⁵. Three muscle-specific products (MyoD^{56,57}, myd⁵⁸ and myogenin⁵⁹) confer a muscle phenotype on various cell types; of these, MyoD and myogenin contain a segment homologous to c-myc.

Aspects of the biology of GF-1 are reminiscent of these regulatory factors. In particular, GF-1 DNA-binding activity is restricted to a particular cell lineage; the cognate DNA-binding site for GF-1 is widely distributed among promoter and enhancer sequences of genes expressed specifically in this lineage; and expression of mRNA encoding the factor is restricted to the lineage in which it acts. On the other hand, the deduced polypeptide sequence of GF-1 predicts two C₂ Zn finger motifs rather than a homoeobox or a myc-related domain. Other proteins with Zn fingers of this general type include the yeast activator Gal4 and members of the steroid receptor family³⁶. The conserved areas of the duplicated Zn fingers domains, particularly at the base or carboxy-terminal to the finger, are likely to be important in determining the specificity of DNA binding⁶⁰. The strong similarity between the finger domains of GF-1 (Fig. 4b) suggests that they may bind the same DNA sequence. This possibility is of interest because it has been shown that a single GF-1 molecule can bind two adjacent TATCT motifs²⁰. The relevance of specific features of the proposed Zn fingers to the

DNA binding specificity and capacity of GF-1 is currently being assessed by site-specific mutagenesis.

As might be anticipated for a regulatory factor critical to gene expression and development of erythroid cells, mouse and human GF-1 proteins appear to be closely related. Direct amino-acid sequencing of purified human protein yielded one peptide with identity to the predicted mouse sequence (Fig. 4b). Furthermore, human protein sequences deduced from clones isolated by cross-hybridization to mouse cDNA support this close relationship (unpublished data). As murine GF-1 cDNA hybridizes to chicken DNA (data not shown), we expect that the chicken protein (Eryf1, ref. 21) will be similarly related at the level of the amino-acid sequence. Southern blot analysis (data not shown) indicates that GF-1 is encoded as a single-copy gene in mice.

Evidence has been provided previously to indicate that GF-1 (ref. 20), Eryf1 (refs 21, 27), or EF-1 (ref. 23) may activate transcription of constructs containing promoters or enhancers bearing its binding site. At present the requirements for transcriptional activation by GF-1 are not defined. In preliminary experiments we have observed modest *trans*-activation of a minimal promoter containing multiple GF-1 binding sites upon cotransfection of heterologous cells (COS or NIH 3T3) with expressible cDNA and reporter constructs, but no activation of more complete promoters or globin genes (unpublished data).

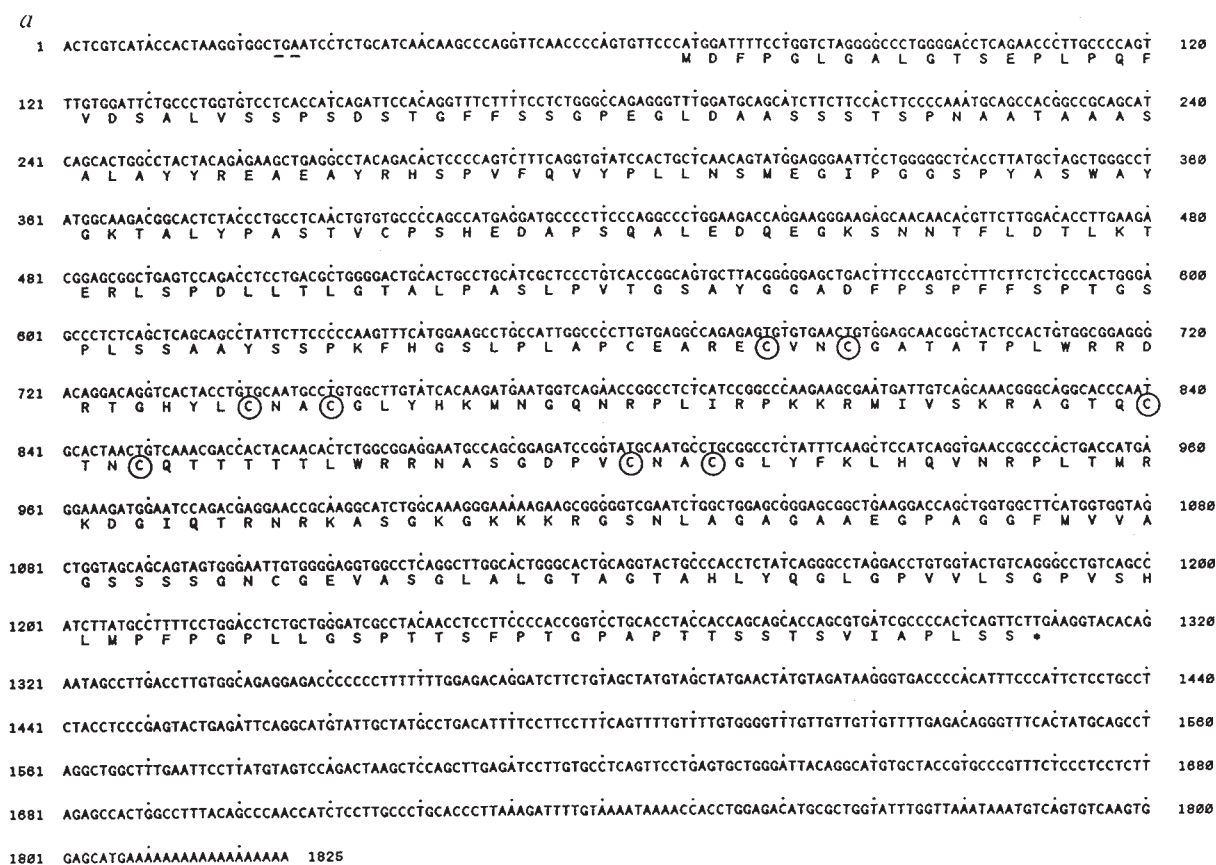
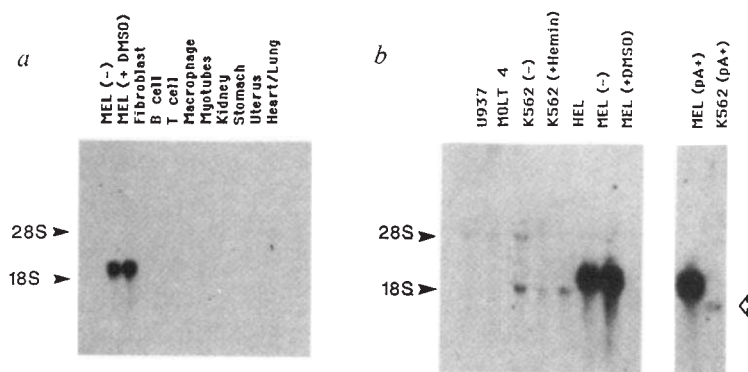


FIG. 4 DNA sequence of GF-1 cDNA and its predicted protein. *a*, The nucleotide sequence of clone number 127 was determined on both DNA strands by the chain-termination method using nested *ExoIII* deletions, specific priming with synthetic oligonucleotides, and directed subcloning into M13 (ref. 68). The 5' extent of clone number 127 was at nucleotide number 46. The additional 5' 46 nucleotides were derived from sequences obtained on two MEL cDNA clones isolated from other libraries. A termination codon upstream of the initiator ATG is underlined with dashes. The paired cysteine residues of the putative Zn fingers are circled. *b*, The repeated Zn finger domains. The predicted amino-acid sequences from 197–250 and 251–304

are aligned. The cysteine pairs are indicated by the bold horizontal lines. Below, the determined amino-acid sequence⁶⁹ of one peptide of purified K562 GF-1 is displayed. This peptide was generated by Endoproteinase Lys-C digestion, followed by separation on HPLC. Amino-acid sequences of human GF-1 will be presented elsewhere.

FIG. 5 Erythroid-specific expression of GF-1 mRNA. *a*, Northern blot analysis of murine RNA samples. MEL (–) and (+DMSO) refer to untreated and induced mouse erythroleukaemia cells (clone 745, GM86). Cell lines: fibroblast (NIH 3T3), B cell (P3X myeloma cells), T cells (BW5127), macrophage (line 2.12). Myotubes, newborn kidney, stomach, uterus and heart/lung were primary tissue samples provided by L. Kunkel. Total cell RNA (20 µg) was electrophoresed per lane. *b*, Cross-hybridization of murine GF-1 cDNA to human mRNA. U937 and MOLT 4 are monocytic and T-cell leukaemia lines, respectively. Total cell RNA (20 µg) was electrophoresed per lane, except for the two lanes to the right where 3 µg of poly(A)⁺ mRNA of MEL and K562 cells were loaded. Open arrow indicates the position of cross-hybridizing, erythroid-specific human mRNA. The filter was washed under non-stringent conditions (2 × SSC at 50 °C). Northern blots were prepared by standard methods⁶⁸ and hybridized with radiolabelled insert of clone number 127.



Whether this reflects the requirement for additional factors, some of which might be erythroid specific, particular features of the constructs used, or potential differences in protein modification in erythroid versus heterologous cells requires further study⁶¹.

As GF-1 appears to form dimers upon interaction with DNA targets containing only a single binding site (for example, the mouse α 1-globin promoter), the possibility exists that protein-protein interactions may join binding sites at different physical locations. One recent model proposes that factors binding to chicken globin promoters and a single enhancer might bring these elements into apposition in a transcriptionally active complex⁶². The widespread distribution of GF-1 binding sites throughout the human β -globin cluster suggests that interactions of GF-1 bound at various sites might influence switching of β -like globin chains during ontogeny and cell differentiation. Such protein-protein interactions might also involve interaction with stage-specific factors, as well as between GF-1 molecules.

The predicted peptide sequence of GF-1 contains three leucine residues, each spaced seven amino acids apart, in the area carboxy-terminal to the Zn finger domains. Although this is reminiscent of the 'leucine zipper' motif⁶³, the region is punctuated with proline residues and, therefore, does not conform to the 'coiled coil' structure recently proposed⁶⁴. Mutagenesis studies will be required to delineate the peptide regions involved in protein-protein interactions of GF-1.

The sites to which GF-1 binds in the human and chicken β -globin gene clusters are associated with DNase I hypersensitivity that is restricted to erythroid progenitors^{9,10,65}. As DNase I sensitivity *in vivo* appears to precede active globin gene transcription^{9,65}, the potential role of GF-1 in promoting these structural alterations in chromatin warrants scrutiny. Finally, an understanding of the molecular basis for the cell-restricted expression of GF-1 RNA should provide important insights into early events in commitment of stem cells to the erythroid lineage. □

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- Maniatis, T., Goodbourn, S. & Fischer, J. A. *Science* **236**, 1237–1245 (1987).
- Lichtsteiner, S., Wuarin, J. & Schibler, U. *Cell* **51**, 963–973 (1987).
- Stamatoyannopoulos, G. & Nienhuis, A. W. *The Molecular Basis of Blood Diseases* (eds Stamatoyannopoulos, G., Nienhuis, A. W., Leder, P. & Majerus, P. W.) 66–105 (Saunders, Philadelphia, 1987).
- Kollas, G., Hurst, J., deBoer, E. & Grosfeld, F. *Nucleic Acids Res.* **15**, 5739–5747 (1987).
- Bodine, D. M. & Ley, T. J. *EMBO J.* **6**, 2997–3004 (1987).
- Townes, T. M., Lingrel, J. B., Chen, H. Y., Brinster, R. L. & Palmiter, R. D. *EMBO J.* **4**, 1715–1723 (1985).
- Hess, J. E., Nickol, J. M., Lieber, M. R. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4312–4316 (1986).
- Behringer, R., Hammer, R. E., Brinster, R. L., Palmiter, R. D. & Townes, T. M. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7056–7060 (1987).
- Tuan, D., Solomon, W., Li, Q. & London, I. M. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6384–6388 (1985).
- Grosfeld, F., van Assendelft, G. B., Greaves, D. R. & Kollas, B. *Cell* **51**, 975–985 (1987).
- Ryan, T. M., Behringer, R. R., Townes, T. M., Palmiter, R. D. & Brinster, R. L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 37–41 (1989).
- Emerson, B. M., Lewis, C. D. & Felsenfeld, G. *Cell* **41**, 21–20 (1985).
- Kemper, B., Jackson, P. D. & Felsenfeld, G. *Molec. cell. Biol.* **7**, 2059–2069 (1987).
- Wall, L., deBoer, E. & Grosfeld, F. *Genes Dev.* **2**, 1089–1100 (1988).
- deBoer, E., Antoniou, M., Mignotte, V., Wall, L. & Grosfeld, F. *EMBO J.* **7**, 4203–4212 (1988).
- Galson, D. L. & Housman, D. E. *Molec. cell. Biol.* **8**, 381–392 (1988).
- Gumucio, D. L. *et al. Molec. cell. Biol.* **8**, 5310–5322 (1988).
- Kim, C. G., Barnhart, K. M. & Sheffery, M. *Molec. cell. Biol.* **8**, 4270–4281 (1988).
- Mignotte, V., Wall, L., de Boer, E., Grosfeld, F. & Romeo, P. *Nucleic Acids Res.* **17**, 37–54 (1989).
- Martin, D. I. K., Tsai, S. & Orkin, S. H. *Nature* **338**, 435–438 (1989).
- Evans, T., Reitman, M. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5976–5980 (1988).
- Knezetic, J. A. & Felsenfeld, G. *Molec. cell. Biol.* **9**, 893–901 (1989).
- Perkins, N. D., Nicolas, R. H., Plumb, M. A. & Goodwin, G. H. *Nucleic Acids Res.* **17**, 1299–1314 (1989).
- Mantovani, R. *et al. Nucleic Acids Res.* **15**, 9349–9364 (1987).
- Mantovani, R. *et al. Nucleic Acids Res.* **16**, 7783–7797 (1988).
- Plumb, M. *et al. Nucleic Acids Res.* **17**, 73–92 (1989).
- Reitman, M. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6267–6271 (1988).
- Kadonaga, J. T. & Tjian, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5889–5893 (1986).
- Strauss, F. & Varshovsky, A. *Cell* **37**, 889–901 (1984).
- Fried, M. & Crothers, D. M. *Nucleic Acids Res.* **9**, 6505–6523 (1981).
- D'Andrea, A. D., Lodish, H. F. & Wong, G. C. *Cell* **57**, 277–285 (1989).
- Plashne, M. *Nature* **335**, 683 (1988).
- Kadonaga, J. T., Carner, K. R., Masiaz, F. R. & Tjian, R. *Cell* **51**, 1079–1090 (1987).
- Norman, C., Runswick, M., Pollock, R. & Treisman, R. *Cell* **55**, 989–1003 (1988).
- Jackson, S. P. & Tjian, R. *Cell* **55**, 125–133 (1988).
- Evans, R. M. & Hollenberg, S. M. *Cell* **52**, 1–3 (1988).

- Schleif, R. *Science* **241**, 1182–1187 (1988).
- Wong, G. G. *et al. Science* **228**, 810–815 (1985).
- Aruffo, A. & Seed, B. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8573–8577 (1988).
- Yamasaki, K. *et al. Science* **241**, 825–828 (1988).
- Sims, J. E. *et al. Science* **241**, 585–589 (1988).
- Singh, H., LeBowitz, J. H., Baldwin, A. S. & Sharp, P. A. *Cell* **52**, 415–423 (1988).
- Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschultz, W. H. & McKnight, S. L. *Genes Dev.* **2**, 801–806 (1988).
- Staudt, L. M. *et al. Science* **241**, 577–580 (1988).
- Sturm, R. A., Das, G. & Herr, W. *Genes Dev.* **2**, 1582–1599 (1988).
- Muller, M. M., Ruppert, S., Schaffner, W. & Matthias, P. *Nature* **336**, 544–551 (1988).
- Wirth, T., Staudt, L. & Baltimore, D. *Nature* **329**, 174–178 (1987).
- Staudt, L. M. *et al. Nature* **323**, 640–643 (1986).
- Scheideit, C., Heguy, A. & Roeder, R. G. *Cell* **51**, 783–793 (1987).
- Scheideit, C. *et al. Nature* **336**, 551–557 (1988).
- Clerc, R. G., Corcoran, L. M., LeBowitz, J. H., Baltimore, D. & Sharp, P. A. *Genes Dev.* **2**, 1570–1581 (1988).
- Bodner, M. *et al. Cell* **55**, 505–518 (1988).
- Castrillo, J., Bodner, M. & Karin, M. *Science* **243**, 814–817 (1989).
- Ingraham, H. A. *et al. Cell* **55**, 519–529 (1988).
- Herr, W. *et al. Genes Dev.* **2**, 1513–1516 (1988).
- Davis, R. L., Weintraub, H. & Lassar, A. B. *Cell* **51**, 987–1000 (1987).
- Tapscott, S. J. *et al. Science* **242**, 405–411 (1988).
- Pinney, D. F., Pearson-White, S. H., Konieczny, S. F., Latham, K. E. & Emerson, C. P. *J. Cell* **53**, 781–793 (1988).
- Wright, W. E., Sassoon, D. A. & Lin, V. K. *Cell* **56**, 607–617 (1989).
- Pfeifer, K., Kim, K., Kogan, S. & Guarente, L. *Cell* **56**, 291–301 (1989).
- Schaffner, W. *Trends Genet.* **5**, 37–39 (1989).
- Choi, O. & Engel, J. D. *Cell* **55**, 17–26 (1988).
- Landschultz, W. H., Johnson, P. F. & McKnight, S. L. *Science* **240**, 1759–1764 (1988).
- O'Shea, E. K., Rutkowski, R. & Kim, P. S. *Science* **243**, 538–542 (1988).
- Forrester, W. C., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G. & Groudine, M. *Nucleic Acids Res.* **15**, 10159–10177 (1987).
- Dignam, J. D., Martin, P. L., Shastri, B. S. & Roeder, R. G. *Meth. Enzym.* **101**, 587–597 (1983).
- Briggs, M. R., Kadonaga, J. T., Bell, S. P. & Tjian, R. *Science* **234**, 47–52 (1986).
- Ausubel, F. M. *et al. in Curr. Protocols molec. Biol.* (Wiley, New York, 1987).
- Matsudaira, P. *J. Biol. Chem.* **262**, 10035–10038 (1987).

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Multiscale periodic structure in the Io wake

Peter R. Smith* & Andrew N. Wright†

THE decametric radio emissions from Jupiter are known to be influenced by the Galilean satellite Io¹. It is believed that the structure in these emissions is associated with the Alfvén-wave wake downstream of Io². However, recent studies^{3,4} have shown that the structure of the wake cannot be as simple as originally thought. Here we present preliminary results from an eigenmode synthesis of the Alfvén waves launched by Io, and find that several important periodicities emerge. Observations of the decametric emissions reveal fine, medium- and large-scale structure. The simulation we present here can provide structure on each of these scales, unlike earlier models.

In 1964 Bigg¹ discovered that the Galilean satellite Io extended an unusual control over decametric radio emissions from Jupiter. It has since become clear that Io is a unique object in the Solar System. It has been suggested that Io has a good electrical conductivity⁵, thus disturbing the magnetoplasma moving past it. In particular, the current induced in the conducting satellite would flow along the flux tube connecting it to Jupiter, thus closing in the jovian ionosphere⁵. The Voyager 1 spacecraft added much to our understanding of the interaction between Io and the jovian magnetosphere. In particular, it was discovered that Io is volcanically active, and matter escaping from the satellite forms a dense torus surrounding the orbit^{6,7}. The presence of the torus further complicates the Io-Jupiter current system. The current will be carried away from the satellite by Alfvén waves⁶ in a manner analogous to the case of artificial satellites⁹. Numerous authors have modelled the structure of these waves in the vicinity of Io^{8,10-12}, which are in excellent agreement with magnetic-field¹³ and plasma^{14,15} observations recorded during the Voyager 1 flyby of Io. Observations of perturbed particles¹⁶ in the Alfvén waves also support this model^{17,18}. It has been suggested that the current-carrying Alfvén waves propagate along the background magnetic field, through the torus density inhomogeneity¹⁹, and suffer efficient reflection from the jovian ionosphere². This would result in multiple subsequent reflections downstream from Io and would produce a steady-state pattern in the satellite rest frame. This is known as the 'multiple Alfvén-wave reflection model'.

More details of the decametric (DAM) wave emission have been revealed by Voyager 1^{20,21}. Figure 1 is an example of the frequency-time spectrographs recorded by Voyager. The most striking feature is the nested arcs. The first panel in Fig. 1 contains arcs shaped like an opening parenthesis, and the second has ones shaped like a closing parenthesis (vertex-early and vertex-late arcs, respectively). When Warwick *et al.*^{20,21} discovered the arcs, they noted that an arc could be produced if DAM emissions are generated at the local electron gyro-

frequency and are then beamed along the surface of a cone whose axis is aligned with the magnetic field. As the field rotates past the observer, an arc is traced out in a plot of frequency against time. Io's Alfvén waves probably provide the source of power upon which the DAM emissions draw. The electron cyclotron maser instability is a likely candidate for the mechanism that converts the Alfvén wave to decametric energy²². Indeed, it is thought that multiple arcs could be produced by the multiple Alfvén-wave reflection model². However, a bouncing Alfvén wave with a round-trip time of 24 min would give rise to an arc spacing in Fig. 1 of about 24°, which is greater than is observed. In addition, the multiple-reflection model yields only one periodicity, whereas several are evident in the data. In the second panel in Fig. 1, the individual arcs have a fine separation of a few degrees in longitude. They also appear to be bunched together in small groups that have a separation of about 17° longitude. Furthermore, the emission appears to die out completely by 1600 UT, suggesting an overall envelope to the emission, called a decametric storm. In other data (Fig. 1 of ref. 23, Fig. 9a, b of ref. 24) the envelope can be seen to extend over an interval of 70° to 190°. The multiple-reflection model will not readily yield small-scale (less than 6°), medium-scale (6° to 60°) and large-scale (greater than 60°) structure. Nevertheless, it has been suggested recently that a model in which each Alfvén-wave bounce excites one of the groups of arcs (rather than an individual arc) will better explain the data²⁵. (It is not clear, however, why each bounce should excite several arcs.) Recent analysis of data has also called into question the Alfvén-wave reflection model on the grounds that the arcs are spaced too closely to be associated with Alfvén-wave reflection²⁶.

Nearly a decade ago, it was noted that Io's Alfvén waves may not propagate completely through the torus in the fashion described by the WKB (Wentzel-Kramers-Brillouin) limit¹⁰. Recent investigations have indeed shown that there is significant reflection (75%) from the torus^{3,4}, so the WKB limit cannot be valid. This means that the multiple-reflection model may yield inaccurate results. If three quarters of the wave power is reflected from the torus, and the remainder is reflected from the ionosphere, it is not immediately obvious that there will be any coherent periodic structure in the Alfvén-wave wake. Here we investigate the evolution of Io's Alfvén waves via a normal-mode synthesis of the disturbance produced in the immediate vicinity of Io. This model is not restricted by the wavelength of the Alfvén wave, thereby extending the range of validity beyond those of previous models.

It is well-known that the toroidal and poloidal motions of field lines in an axisymmetric magnetosphere become decoupled in a variety of highly symmetric or unsymmetric situations^{27,28}. They also decouple in the absence of a parallel-field perturbation²⁹, which occurs when $\nabla \cdot (\mathbf{u}_\perp B_0^2) = 0$ (in a force-free background field, $\mathbf{B}_0$). (Here $\mathbf{u}_\perp$ is the perturbation plasma velocity perpendicular to $\mathbf{B}_0$.) Voyager 1 observations show that the field perturbation at Io has a negligible parallel component¹³. Pioneer 10 measurements of the wake downstream from Io show that

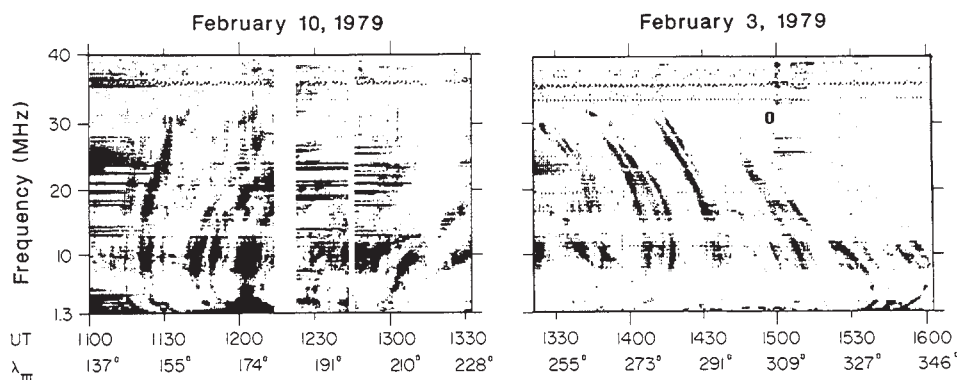


FIG. 1 Plots of decametric emission frequency against universal time and space-craft longitude, as recorded by Voyager 1. Dark patches correspond to strong emission. Note the nested arc-like structures. (After ref. 2.)

the parallel-field perturbation there is still negligible³⁰. Based on these data, and the fact that one would expect $\nabla \cdot (\mathbf{u}_\perp B_0^2)$ to be negligible for an Alfvén wave localized across the field, we shall decouple the toroidal and poloidal motions. For a dipole background field ($\mathbf{B}_0$) the Sturm-Liouville equation for the toroidal magnetic field perturbation is³

$$\frac{\partial}{\partial \lambda} \left\{ [\rho_n \cos^3(\lambda)]^{-1} \frac{\partial b^*}{\partial \lambda} \right\} + \omega^2 L_I^2 \cos(\lambda) b^* = 0$$

where $b^* = b \cos^3 \lambda$, λ is the colatitude, and the derivative is taken along the field line. b and ρ_n are, respectively, the field perturbation and density normalized by the background equatorial values. L_I is the value of the L shell of Io (equal to 5.91). The density profile used has a scale length of one jovian radius and is similar to that used in previously modelling³. The boundary condition of no velocity perturbation at the ends of the field lines ($\lambda = \pm 65^\circ$) enables one to calculate the eigenfrequencies (ω) of the toroidal normal modes. The north- and south-bound Alfvén waves launched by Io have been sketched previously (Fig. 11 of ref. 12). For simplicity we shall model their shape as two gaussians of half-width 2° . We shall also simplify the synthesis by running the perturbations back in time until the north- and south-bound waves overlap each other completely.

The time units employed here are the ratio of one jovian radius (7.14×10^4 km) to the equatorial Alfvén speed

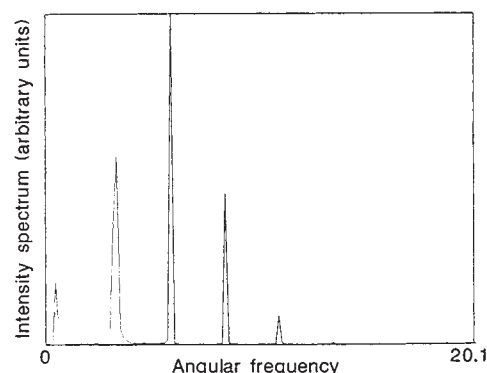


FIG. 3 The power spectrum of the fluctuating magnetic disturbance observed at $\lambda = 57^\circ$, in the plasma rest frame. The peaks correspond to the eigenfrequencies discussed in the text.

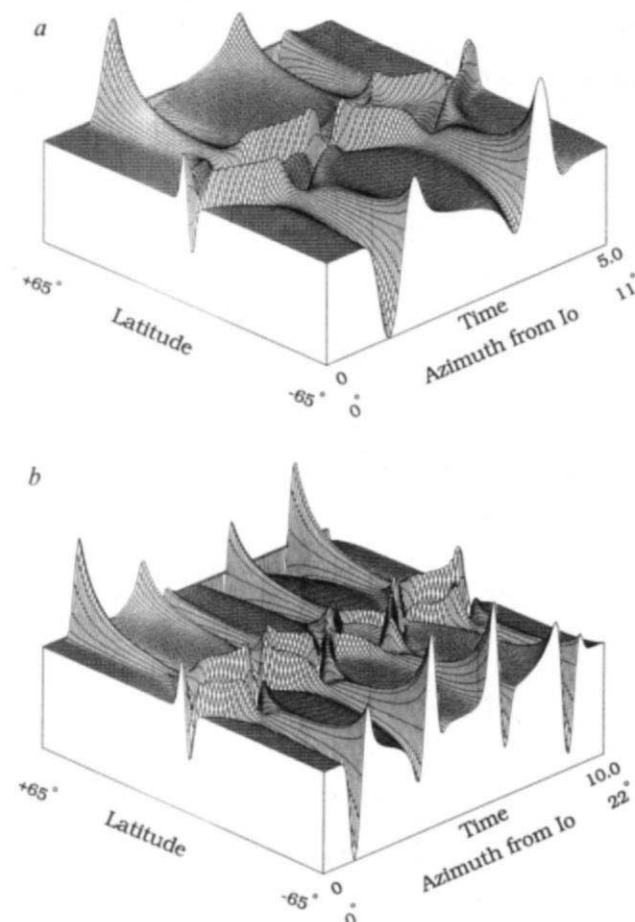


FIG. 2 Surface plot of the variation of toroidal magnetic-field disturbance produced by Io with latitude and azimuth downstream from Io (or equivalently, with time). The height of the surface from the ambient level is proportional to the toroidal magnetic-field perturbation. The perturbation at $(\lambda, t=0)$ separates into two waves propagating toward $\lambda = \pm 65^\circ$ and represents the north- and south-bound waves excited by Io. *a*, High time resolution, *b*, Longer-term evolution.

(250 km s^{-1}), equal to 4.76 min. The angular frequencies of the fundamental and first few harmonics are 0.5071, 1.9082, 3.2159 and 4.5009. The higher harmonics have eigenfrequencies very close to the ray-tracing estimates (the third harmonic differs by 10%, and the twentieth by only 1%). The shape of the field eigenmodes exhibit a structured, almost sinusoidal, variation in the vicinity of the torus and a smooth, unstructured shape outside. This is due to the rapid increase in Alfvén speed outside the torus. The first 40 modes have been used to synthesize the initial condition described earlier. The variation of the magnetic-field perturbation with latitude and time (or equivalently azimuth downstream from Io) is shown in Fig. 2. Figure 2*a* shows the evolution in high time resolution, and Fig. 2*b* shows the long-term development. At $t = 0$ the bipolar double-gaussian wave-packet is clearly visible. As time increases, the packet splits into two waves that propagate in opposite directions, representing the north- and south-bound waves generated by Io (Fig. 2*a*). The plot shows features reminiscent of wave reflection at the ionosphere and propagation toward the opposite hemisphere, but we shall see below that such a description is inaccurate. There is a strong oscillatory motion in Fig. 2 with a period of about 2 time units. The longer-term development also shows a broad modulation, as the ambient perturbation at the end of the field line gradually rises and falls.

To study the periodicities in the wake, the power spectrum of the perturbation at a given latitude was calculated. Figure 3 is the power spectrum for a latitude of 57° (expected emission ~ 25 MHz) and several dominant frequencies are found (as one would expect from a normal-mode synthesis). The time periods (in the plasma rest frame) of these frequencies and the corresponding longitude interval in a plot such as Fig. 1 are 12.3, 1.95, 1.09, 0.756 and 0.580 time units (58.6, 9.3, 5.19, 3.60 and 2.76 min) and 116.4, 18.5, 10.3, 7.15 and 5.48 degrees longitude, respectively. As each oscillation corresponds to an upward and downward current, it may be possible to generate structure in the decametric emissions on half the size of these intervals also.

The poloidal evolution is quantitatively very similar to the toroidal one, so we shall not show the results here. It is interesting to note that the WKB or ray-tracing period (24.1 min round-trip time, corresponding to a 12-min oscillation or 24° longitude period) is absent, suggesting that there is no WKB behaviour in Fig. 2.

In conclusion, an eigenmode synthesis of the perturbation produced by Io reveals that the Io wake will have periodic structure. The preliminary results presented here show how the wake can supply small-scale structure (such as that between adjacent arcs in Fig. 1), medium-scale structure (the grouping of arcs), and even the large-scale structure (such as the gradual brightening and fading of DAM emissions) that constitutes a decametric storm. We have revised the basic multiple-reflection

model so as to give a realistic picture of decoupled field-line motion, with encouraging results. The next step will be to consider the problem of antisymmetric perturbations, such as those excited when Io is off the equator. Further progress can be made by a non-trivial analysis of field-line coupling with a compressional field component. □

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1. Bigg, E. K. *Nature* **203**, 1008-1010 (1964).
2. Gurnett, D. A. & Goertz, C. K. *J. geophys. Res.* **86**, 717-722 (1981).
3. Wright, A. N. *J. geophys. Res.* **92**, 9963-9970 (1987).
4. Wright, A. N. & Schwartz, S. J. *J. geophys. Res.* (in the press).
5. Goldreich, P. & Lynden-Bell, D. *Astrophys. J.* **156**, 59-78 (1969).
6. Bridge, H. S. *et al. Science* **204**, 987-991 (1979).
7. Broadfoot, A. L. *et al. Science* **204**, 979-982 (1979).
8. Goertz, C. K. & Deift, P. A. *Planet. Space Sci.* **21**, 1399-1415 (1973).
9. Drell, S. D., Foley, H. M. & Ruderman, M. A. *J. geophys. Res.* **70**, 3131-3145 (1965).
10. Neubauer, F. M. *J. geophys. Res.* **85**, 1171-1178 (1980).
11. Southwood, D. J., Kivelson, M. G., Walker, R. J. & Slavin, J. A. *J. geophys. Res.* **85**, 5959-5968 (1980).
12. Wright, A. N. & Southwood, D. J. *J. geophys. Res.* **92**, 1167-1175 (1987).
13. Acuña, M. H., Neubauer, F. M. & Ness, N. F. *J. geophys. Res.* **86**, 8513-8522 (1981).
14. Belcher, J. W., Goertz, C. K., Sullivan, J. D. & Acuña, M. H. *J. geophys. Res.* **86**, 8508-8512 (1981).
15. Barnett, A. S. *J. geophys. Res.* **91**, 3011-3019 (1986).
16. Lanzerotti, L. J. *et al. J. geophys. Res.* **86**, 8491-8496 (1981).
17. Schulz, M. & Evliatar, A. *Astrophys. J.* **211**, L149-L154 (1977).
18. Wright, A. N. *J. geophys. Res.* **92**, 3155-3164 (1987).
19. Goertz, C. K. *J. geophys. Res.* **85**, 2949-2956 (1980).
20. Warwick, J. W. *et al. Science* **204**, 995-998 (1979).
21. Warwick, J. W. *et al. Science* **206**, 991-995 (1979).
22. Goldstein, M. L. & Goertz, C. K. in *Physics of the Jovian Magnetosphere* (ed. Dessler, A. J.) 317-352 (Cambridge University Press, New York, 1983).
23. Leblanc, Y. & Genova, F. *J. geophys. Res.* **86**, 8564-8568 (1981).
24. Leblanc, Y. *J. geophys. Res.* **86**, 8546-8560 (1981).
25. Bagenal, F. & Leblanc, Y. *Astr. Astrophys.* **197**, 311-319 (1988).
26. Staelin, D. H., Garnavich, P. M. & Leblanc, Y. *J. geophys. Res.* **93**, 3942-3948 (1988).
27. Radoski, H. R. *J. geophys. Res.* **72**, 4026-4027 (1967).
28. Dungey, J. W. *Hydromagnetic waves, Physics of Geomagnetic Phenomena* Vol. 2 (eds Matsushita, S. & Campbell, W. H.) 913-934 (Academic Press, London, 1967).
29. Singer, H. J., Southwood, D. J., Walker, R. J. & Kivelson, M. G. *J. geophys. Res.* **86**, 4589-4596 (1981).
30. Walker, R. J. & Kivelson, M. G. *Geophys. Res. Lett.* **8**, 1281-1284 (1981).

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Hydroformylation by supported aqueous-phase catalysis: a new class of heterogeneous catalysts

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HOMOGENEOUS catalysts typically operate at relatively mild temperatures and exhibit high activities and selectivities¹. However, it has long been recognized that many homogeneous reactions are not commercially viable because of problems of catalyst recovery. Thus, significant efforts have centred around the immobilization of the organometallic species responsible for catalysis². At best, the performance of such heterogeneous catalysts only approximate those of their homogeneous counterparts. Here we describe a novel family of heterogeneous catalysts, denoted supported aqueous-phase catalysts (SAPCs), designed to facilitate chemical reactions at the interface of two liquids. These catalysts consist of a water-soluble organometallic complex dissolved in a film of water which is supported on a high-surface-area hydrophilic solid. Reactions of liquid-phase organic reactants take place at the water-organic interface. Heterogeneous hydroformylation of alkenes by SAPCs containing water-soluble organometallic rhodium complexes is used to illustrate the efficacy of these new catalysts.

Hydroformylation involves the reaction of carbon monoxide and hydrogen with an alkene to produce aldehydes, a reaction that does not proceed in the absence of a catalyst. It is the oldest

and most widely used homogeneous catalytic reaction of alkenes¹. No heterogeneous hydroformylation catalysts are used at present for liquid-phase conversions, primarily because of problems arising from catalyst loss to the product-containing phase.

Over the past decade, the interest in homogeneous catalysis using water-soluble, transition-metal complexes has increased significantly. One reason for this is the commercialization in 1984 of butanal production from propylene using a water-soluble rhodium phosphine complex³. Like many metals contained in biological systems, such as magnesium in chlorophyll and iron in haemoglobin, the rhodium-based catalyst contains ligands that ensure hydrophilic properties; coordination of the metal maintains the rhodium in the aqueous phase³ while the local environment at the metal remains hydrophobic in character. It is suggested that the hydroformylation of propylene occurs homogeneously in the aqueous phase and that the butanal so produced forms a second phase because of its low solubility in water. Here we demonstrate hydroformylation using a new class of heterogeneous catalysts, supported aqueous-phase catalysts.

Supported liquid-phase catalysts (SLPCs) have been reported previously⁴. The system described here differs significantly from traditional SLPCs. First, SLPCs are designed for gas-phase reactants and suffer from extraction of the supported phase when liquids are used as substrates. Second, the supported liquid phase must have a low vapour pressure and must be hydrophobic in order to dissolve traditional organometallic catalysts. Supported aqueous-phase catalysts (SAPCs) are designed to work on liquid reactants, with the low-boiling-point supported phase—water—maintained by using a hydrophilic support. Immobilization of the catalyst is accomplished by means of the insolubility of the rhodium complex in the organic (reactant-bearing) media.

Figure 1 illustrates the general SACP scheme. A high-surface-area, hydrophilic support, such as silica, is contacted with a water-soluble organometallic complex by aqueous-phase impregnation. Upon evacuation of the water phase used for impregnation, the organometallic complex is distributed over the surface of the support. Exposure to vapour-phase water for fixed times allows precise amounts of water to condense on the solid surface. As the water-soluble complexes are typically hygroscopic, they facilitate condensation of water vapour. The solid, coated by an aqueous film containing the catalytic species, is placed in contact with an immiscible, liquid, organic phase containing reactant molecules. Reactants diffuse from the bulk

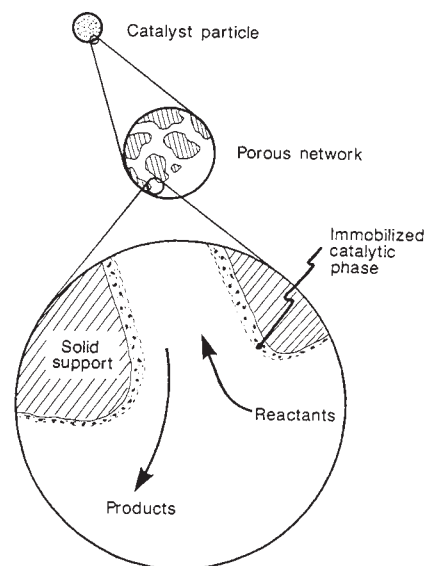


FIG. 1 Schematic diagram of a supported aqueous-phase catalyst.

organic phase into the porous solid, react at the water-organic interface, and products diffuse back out to the bulk organic phase. In addition to the immobilization of the organometallic species, the advantages of this novel system include the high surface area provided by the solid (and thus the increased interfacial area between the organic and water phases), and the possibility of selectivity variations from bulk equilibrium product distributions through the effect of the interface. The first application of a SAPC system is described below.

Narrow-pore-size-distribution silica, CPG-240, was impregnated with $\text{HRh}(\text{CO})[\text{P}(m\text{-C}_6\text{H}_4\text{SO}_3\text{Na})_3]_3$ ($\text{P}(m\text{-C}_6\text{H}_4\text{SO}_3\text{Na})_3$: trisodium salt of tri(*m*-sulphophenyl) phosphine) and free $\text{P}(m\text{-C}_6\text{H}_4\text{SO}_3\text{Na})_3$ in a 1:1.3 molar ratio. ^{31}P NMR and Fourier-transform infrared studies reveal the presence of $\text{HRh}(\text{CO})[\text{P}(m\text{-C}_6\text{H}_4\text{SO}_3\text{Na})_3]_3$ after deposition onto the silica support and condensation of vapour-phase water to form the aqueous film. As normal liquid-phase ^{31}P NMR was used, the rhodium complexes must be somewhat mobile within the water layer in order that detection be possible. Complete details of catalyst preparation and analyses are available⁵. Electron microprobe analysis reveals that the rhodium is distributed uniformly over the silica. The catalyst composition is typically 0.18 g Rh per 100 g CPG-240 and 2.9 g H_2O per 100 g catalyst. The amount of water in the catalyst was varied from 2.9 to 67 wt% and was found to greatly influence the catalytic activity (see below). The optimum water content was around 8–9 wt%.

Oleyl alcohol was the first reactant tested, because neither it nor its products are water-soluble. Also, the double bond in oleyl alcohol is internal, and thus it is unlikely that it can bind to the rhodium complex at the interface and then migrate into the aqueous layer. A terminal alkene could, however, allow the rhodium complex to diffuse a short distance away from the water-organic interface. Typical batch-reaction conditions were: 0.002 g Rh per 1 g oleyl alcohol, organic phase was 25 vol% oleyl alcohol in cyclohexane, molar ratio $\text{CO}/\text{H}_2 = 1$, pressure was 5.08 MPa, temperature was 373 K and reaction time was 5.5 h. Oleyl alcohol was successfully converted to aldehydes (96.6%) under these conditions. No other products were detected.

The SAPCs were demonstrated to be stable to decompression and recycling in the following way. After a catalytic run, the pressure was reduced and the solid catalyst particles were collected by filtration. These particles effected the hydroformylation of oleyl alcohol in subsequent batch reactions.

The hydroformylation reaction was attempted in the absence of the SAPC, using the filtrate plus additional oleyl alcohol at conditions identical to those above; no further conversion was detected. To the same filtered solution, 1-hexene was added and the reactor pressurized with H_2 (no CO) to 0.51 MPa at 373 K for 2 h; no hydrogenation products were observed. These tests indicate that rhodium is not leached into the organic phase. We have shown previously^{6,7} that a sensitive test for rhodium leaching in a liquid-phase hydroformylation reaction is the presence of catalytic activity in the organic phase after filtration. It is possible for a supported catalyst to show no loss of activity through recycling, and to show no loss of rhodium (by routinely available analytical methods), yet to leach catalytically active amounts of rhodium into the substrate phase^{6,7}.

The following experiment further supported the inference that the reaction chemistry is taking place at the interface. CPG-350 (with a surface area less than that of CPG-240) was used as the supporting silica, and the amount of SAPC used in the hydroformylation reaction was fixed such that the total surface area in the reactor was the same as for CPG-240. Reaction results were consistent with the premise that the conversion is proportional to the interfacial area.

This reaction system is very sensitive to water content. When $\text{HRh}(\text{CO})[\text{P}(m\text{-C}_6\text{H}_4\text{SO}_3\text{Na})_3]_3$ is dissolved in water and contacted with oleyl alcohol in cyclohexane as solvent (no solid present), no hydroformylation is observed for $\text{CO}/\text{H}_2 = 1$, $P =$

5.08 MPa and $T = 373$ K. The aqueous solution turns from bright yellow to dark brown, most probably indicating decomposition of the rhodium complex. Also, at high water loadings, (~67 wt%), the SAPC changes colour from yellow to brown and is not active. Thus, at low water levels the rhodium complex remains stable on the SAPC. The reasons for the stability of the complex are now under investigation.

Among other substrates, 1-octene and dicyclopentadiene were hydroformylated. The hydroformylation of 1-octene yielded a nonanal/2-methyl octanal (n/b) ratio ranging from 1.8 to 2.9 depending on the water content of the catalyst and on the ligand/rhodium ratio (which varied from 7 to 30).

Reactions that occur at an interface can differ from those proceeding in the bulk fluid phase as a result of the orientation of molecules at the interface⁸. The geometrical arrangement at the interface can lead to alterations in reaction rate, equilibrium and selectivity. Examples of such reactions are emulsion polymerization and hydrolysis of fats. We are now investigating the possibility of these effects with SAPCs. □

Received 21 February; accepted 2 May 1989.

1. Parshall, G. W. *Homogeneous Catalysis* (Wiley, New York, 1980).
2. Bailey, D. C. & Langer, J. H. *Chem. Rev.* **81**, 109–148 (1981).
3. Kuntz, E. G. *Chemtech* **17**, 570–575 (1987).
4. Rony, P. R. & Roth, J. F. US Patent 3,855,307 (1974).
5. Arhancet, J. P. thesis, Virginia Polytechnic Institute and State University (1989).
6. Davis, R. J., Rossin, J. A. & Davis, M. E. *J. Catal.* **98**, 477–486 (1986).
7. Taylor, D. F., Hanson, B. E. & Davis, M. E. *Inorg. Chim. Acta* **128**, 55–60 (1987).
8. Davies, J. T. *Adv. Catal.* **6**, 1–65 (1954).

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Isotopic tracers of lead contamination in the Great Lakes

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FLUXES of lead to the Great Lakes are dominated by atmospheric depositions of industrial lead, which account for ~64% of the lead inputs to Lake Ontario and >90% of the inputs to Lake Superior¹. It has recently been demonstrated that lead aerosols in the Great Lakes region may be identified by the contrasting $^{206}\text{Pb}/^{207}\text{Pb}$ ratios of industrial leads from the United States (1.221 ± 0.009) and Canada (1.151 ± 0.010)². Here we show that those ratios may also be used to identify and trace industrial lead inputs to the Great Lakes. These corroborate spatial gradients in lead concentrations in surface waters, which range from 290 pmol kg^{-1} in Hamilton Harbour to <10 pmol kg^{-1} in the central waters of Lake Ontario. The latter concentrations and corresponding residence-time estimates, which are both an order of magnitude lower than previously reported, indicate that lead is rapidly scavenged in the epilimnion during periods of high primary productivity. We find that industrial lead from Canada and the United States are the two principal sources of lead contamination in the Great Lakes.

Lead concentrations and isotopic compositions in the Great Lakes were measured with ultra-clean techniques developed for seawater analyses³. Samples (2 l) of epilimnetic (1 m depth) waters in Lake Ontario and Lake Erie were collected from a raft in August 1987, at Environment Canada monitoring stations for the Great Lakes Surveillance Program. Elemental lead concentrations and isotopic compositions were measured in filtered

(<0.4 μm) samples by thermal-ionization mass spectrometry⁴. Particulate concentrations were not measured in this preliminary study, because of their inherent variability. The analytical detection limit was 0.1 pmol. The fractionation correction derived from concurrent calibrations of SRM 981 was $0.12 \pm 0.02\%$. The sum of contaminant lead (sampling, storage and analytical) was ≤ 1 pmol, with an isotopic composition analogous to that of urban aerosols in the United States ($^{206}\text{Pb}/^{207}\text{Pb} = 1.22$). The mass spectrometric measurements of lead concentrations were corroborated with graphite-furnace atomic-absorption spectrometry measurements following an organic extraction^{5,6}. Details of this intercalibration, as well as complementary trace-element (Cd, Cu, Zn) concentrations, will be reported elsewhere⁶.

The predominance of anthropogenic lead inputs to the Great Lakes was evidenced by the distribution of dissolved lead concentrations in Lake Ontario (Table 1). Concentrations were elevated along the western shore near primary sources of industrial lead inputs to the lake (atmospheric emissions, $3.79 \times 10^5 \text{ kg yr}^{-1}$; industrial effluents, $1.7 \times 10^4 \text{ kg yr}^{-1}$; municipal effluents, $1.42 \times 10^5 \text{ kg yr}^{-1}$; and other anthropogenic sources, $5.4 \times 10^4 \text{ kg yr}^{-1}$)¹. Unusually high concentrations ($291 \pm 13 \text{ pmol kg}^{-1}$) were measured in Hamilton Harbour, which receives municipal and industrial effluents with elevated lead concentrations of 410 nmol kg^{-1} and $1.5 \mu\text{mol kg}^{-1}$ respectively¹. The drainage of industrial lead from the other Great Lakes into the western basin of Lake Ontario may have also contributed to the relatively high lead concentrations ($30\text{--}97 \text{ pmol kg}^{-1}$) in that area (Fig. 1a). In contrast, lead concentrations in most offshore surface waters in the central and eastern

basins of the lake ($6\text{--}22 \text{ pmol kg}^{-1}$) were comparable with lead concentrations of remote oceanic surface waters⁴.

The distribution of lead concentrations in Lake Erie was analogous to that in Lake Ontario (Table 1). Relatively low concentrations (15 and 28 pmol kg^{-1}) were found in offshore waters in the northern and central regions of the lake, whereas higher concentrations (92 to 135 pmol kg^{-1}) were observed in the southern region of the lake (Fig. 1a). This distribution corresponded to the location of primary sources of lead inputs to the lake (atmospheric deposition, $7.54 \times 10^5 \text{ kg yr}^{-1}$; industrial effluents, $2.2 \times 10^4 \text{ kg yr}^{-1}$; municipal effluents, $2.16 \times 10^5 \text{ kg yr}^{-1}$; and other anthropogenic sources, $9.9 \times 10^4 \text{ kg yr}^{-1}$)¹.

The annual flux of industrial lead to Lake Erie ($1.09 \times 10^6 \text{ kg yr}^{-1}$) is nearly twice that to Lake Ontario ($5.92 \times 10^5 \text{ kg yr}^{-1}$). Conversely, the volume of water in Lake Ontario ($1.63 \times 10^3 \text{ km}^3$) is nearly three times greater than that of Lake Erie ($4.68 \times 10^2 \text{ km}^3$). Presumably, those differences contribute to the apparent difference in average lead concentrations of offshore surface waters in Lake Erie (77 pmol kg^{-1}) and Lake Ontario (30 pmol kg^{-1}).

The anthropogenic origins of lead in Lake Ontario indicated by the preceding lead concentration gradients were corroborated by stable lead isotopic composition measurements (Table 1). Most of the $^{206}\text{Pb}/^{207}\text{Pb}$ ratios ($1.147\text{--}1.160$) in the western and central basins of Lake Ontario were within the normal range of aerosols in the Toronto metropolitan area ($^{206}\text{Pb}/^{207}\text{Pb} = 1.151 \pm 0.010$)². The $^{206}\text{Pb}/^{207}\text{Pb}$ ratios ($1.180\text{--}1.184$) at two locations in the eastern basin, as well as in Hamilton Harbour, were intermediate between those of urban aerosols in Canada ($^{206}\text{Pb}/^{207}\text{Pb} = 1.151 \pm 0.010$) and the United States

TABLE 1 Dissolved ($\leq 0.4 \mu\text{m}$) lead concentrations (pmol kg^{-1}) and isotopic compositions in surface waters of Lake Ontario and Lake Erie (July 1987)

Station number	Location (lat./long.)	Pb ($\bar{x} \pm s$)	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$ ($\bar{x} \pm 95\% \text{ c.l.}$)	$^{208}\text{Pb}/^{204}\text{Pb}$	$^{206}\text{Pb}/^{207}\text{Pb}$
Lake Ontario						
8	43°37'24" N 79°27'12" W	97.1 $\pm$ 39.4	18.00 $\pm$ 0.02	15.55 $\pm$ 0.02	37.58 $\pm$ 0.05	1.1577 $\pm$ 0.0002
12	43°30'12" N 79°21'12" W	29.7 $\pm$ 15.0	18.05 $\pm$ 0.01	15.60 $\pm$ 0.01	37.73 $\pm$ 0.03	1.1568 $\pm$ 0.0003
41	43°43'00" N 78°01'36" W	10.4 $\pm$ 1.0	17.99 $\pm$ 0.02	15.59 $\pm$ 0.02	37.60 $\pm$ 0.04	1.1541 $\pm$ 0.0006
45	43°49'12" N 77°47'00" W	35.6 $\pm$ 1.0	18.04 $\pm$ 0.01	15.57 $\pm$ 0.01	37.67 $\pm$ 0.03	1.1588 $\pm$ 0.0003
49	43°46'18" N 77°26'18" W	48.0 $\pm$ 32.2	17.85 $\pm$ 0.01	15.57 $\pm$ 0.01	37.54 $\pm$ 0.03	1.1463 $\pm$ 0.0003
64	43°31'30" N 76°53'36" W	9.2 $\pm$ 4.8	17.49 $\pm$ 0.04	15.54 $\pm$ 0.02	37.13 $\pm$ 0.11	1.1258 $\pm$ 0.0033
71	43°28'36" N 76°31'36" W	19.7 $\pm$ 8.7	17.88 $\pm$ 0.02	15.58 $\pm$ 0.01	37.53 $\pm$ 0.03	1.1483 $\pm$ 0.0005
81	44°01'00" N 76°40'18" W	19.4 $\pm$ 7.9	18.27 $\pm$ 0.02	15.59 $\pm$ 0.01	37.89 $\pm$ 0.04	1.1827 $\pm$ 0.0003
89	43°41'54" N 76°25'00" W	6.5 $\pm$ 3.7	18.19 $\pm$ 0.03	15.59 $\pm$ 0.02	37.76 $\pm$ 0.05	1.1671 $\pm$ 0.0007
90	43°55'12" N 78°18'24" W	22.0 $\pm$ 0.3	18.41 $\pm$ 0.11	15.61 $\pm$ 0.11	38.09 $\pm$ 0.21	1.1794 $\pm$ 0.0027
104	43°17'00" N 79°50'00" W	291.5 $\pm$ 13.4	18.21 $\pm$ 0.01	15.59 $\pm$ 0.01	37.74 $\pm$ 0.02	1.1681 $\pm$ 0.0003
Lake Erie						
23	42°29'52" N 79°53'58" W	27.7 $\pm$ 0.6	18.02 $\pm$ 0.02	15.57 $\pm$ 0.02	37.63 $\pm$ 0.06	1.1571 $\pm$ 0.0004
84	41°55'35" N 81°39'43" W	114.5 $\pm$ 30.4	18.78 $\pm$ 0.01	15.63 $\pm$ 0.01	38.27 $\pm$ 0.02	1.2016 $\pm$ 0.0004
85	41°52'14" N 83°05'59" W	15.4 $\pm$ 3.2	18.54 $\pm$ 0.02	15.62 $\pm$ 0.02	38.09 $\pm$ 0.04	1.1863 $\pm$ 0.0004
357	41°49'44" N 82°58'13" W	91.6 $\pm$ 10.0	18.62 $\pm$ 0.01	15.65 $\pm$ 0.01	38.19 $\pm$ 0.03	1.1902 $\pm$ 0.0003
358	41°53'38" N 82°51'59" W	135.0 $\pm$ 24.0	18.73 $\pm$ 0.01	15.62 $\pm$ 0.02	38.21 $\pm$ 0.02	1.1989 $\pm$ 0.0004

($^{206}\text{Pb}/^{207}\text{Pb} = 1.221 \pm 0.009$)². Spatial variations in $^{206}\text{Pb}/^{207}\text{Pb}$ ratios in surface waters of Lake Ontario (Fig. 1b) conformed with previous measurements of temporal variations in the $^{206}\text{Pb}/^{207}\text{Pb}$ ratios of plumbiferous aerosols along the northern shore of the lake². Those aerosols contained Canadian industrial leads when the winds were from the north ($1.10 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.16$) and a mixture of industrial leads from Canada and the United States when the winds were from the south ($^{206}\text{Pb}/^{207}\text{Pb} = 1.19$).

Lead isotopic compositions of surface waters in Lake Erie were consistent with those in Lake Ontario (Table 1). The $^{206}\text{Pb}/^{207}\text{Pb}$ ratio of surface waters in the northern region of Lake Erie (1.157) was similar to those of waters in western Lake Ontario and urban aerosols in Canada, whereas the $^{206}\text{Pb}/^{207}\text{Pb}$ ratios in the central and southern regions of the lake (1.186–1.201) were closer to those of urban aerosols in the United States². Again, this spatial variation (Fig. 1b) conformed with previous measurements of the temporal variations in plumbiferous aerosols along the northern shore of Lake Erie². Those aerosols contained Canadian industrial leads when the winds were from the north-east ($1.14 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.16$) and a mixture of industrial leads from Canada and the United States when the winds were from the south ($1.18 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.25$). Therefore, the distribution of lead isotopic compositions in surface waters of both Lake Erie and Lake Ontario reflected the atmospheric dispersion of industrial leads from the United States and Canada.

Ratios of radiogenic lead isotopes (^{206}Pb , ^{207}Pb and ^{208}Pb) to ^{204}Pb in Lake Ontario and Lake Erie, as well as the $^{206}\text{Pb}/^{207}\text{Pb}$

ratios used to characterize anthropogenic lead aerosols in that region², are listed in Table 1. Simple linear regressions among all isotopic compositions were highly significant ($p < 0.01$). Lines of best fit for three-isotope, two-dimensional plots were determined using a least-squares program, which included uncertainties on all ratios and accounted for correlated errors⁷. Reduced χ^2 values (1.6–2.7) confirmed that the predominant components of lead in the Great Lakes were industrial leads from Canada and the United States. This corroborates previous mass-balance calculations which indicated that anthropogenic lead inputs from Canada and the United States account for more than 99% of the total lead flux to the Great Lakes¹.

The low concentrations of lead in Lake Erie and Lake Ontario, as well as spatial variations in lead concentrations and isotopic compositions, suggest that lead is rapidly scavenged from the epilimnion during the periods of high primary productivity. The steady-state residence time of dissolved lead in surface waters of Lake Ontario during the summer is estimated to be 4 days, based on the lake's surface area (19,684 km²), thermocline depth (15 m), atmospheric lead input (3.79×10^5 kg yr⁻¹), and the labile fraction (43%) of lead aerosols⁸. The corresponding residence time in Lake Erie is estimated to be 7 days, based on the lake's surface area (26,900 km²), thermocline depth (15 m) and atmospheric lead flux (7.54×10^5 kg yr⁻¹). These approximations are an order of magnitude lower than previous estimates based on reports of higher lead concentrations in the Great Lakes.

However, the new estimates are consistent with those for Lake Constance, where the residence times of lead and particles are comparable⁹. This is attributed to the high particle-water partition coefficient (10^3 m³ kg⁻¹) of lead in Lake Constance, which is similar to the partition coefficient of lead in the Mississippi River¹⁰. These evidence the rapid scavenging of dissolved lead through the formation of nuclei/particle surface site complexes, including dissolved organic carbon peptization and particulate organic carbon (POC) coagulation¹¹. Those processes control the distribution of lead within aquatic systems and are particularly important in waters with high organic loadings. This should include the Great Lakes during summer algal blooms, when POC production is intense¹².

Lead concentrations and isotopic compositions in surface waters of Lake Ontario and Lake Erie show, therefore, that essentially all of that lead is derived from industrial sources in Canada and the United States. These measurements may be used to monitor projected declines of lead concentrations in the Great Lakes corresponding to decreases in atmospheric emissions of lead in gasoline, as they have in the North Atlantic¹³. Other atmospheric emissions of lead from Canada and the United States (municipal waste incineration, 2.8×10^6 kg yr⁻¹; industrial processes, 2.3×10^6 kg yr⁻¹; coal combustion, 7.78×10^5 kg yr⁻¹; and miscellaneous sources, 2.7×10^4 kg yr⁻¹)^{14,15}, as well as direct inputs from waste-water discharges and solid waste disposal, may also be identified by their isotopic composition, as they have in the north-east Pacific^{16,17}. Finally, lead isotopic compositions may be used to trace the sources of associated contaminants, including acid-rain precursors, in the Great Lakes^{18,19}. □

Received 28 December 1988; accepted 11 April 1989.

1. Nriagu, J. O. in *The Role of the Oceans as a Waste Disposal*, 441–468 (Reidel, Dordrecht, 1986).
2. Sturges, W. T. & Barrie, L. A. *Nature* **329**, 144–146 (1987).
3. Flegal, A. R. & Patterson, C. C. *Earth planet. Sci. Lett.* **64**, 19–32 (1983).
4. Flegal, A. R. & Stukas, V. J. *Mar. Chem.* **22**, 163–177 (1987).
5. Bruland, K. W., Coale, K. H. & Mart, L. *Mar. Chem.* **17**, 285–300 (1985).
6. Coale, K. H. & Flegal, A. R. *Sci. tot. Envir.* (in the press).
7. Hudson, B. thesis, Washington Univ. (1981).
8. Lum, K. R., Kokotich, E. A. & Schroeder, W. H. *Sci. tot. Envir.* **63**, 161–173 (1987).
9. Sigg, L. in *Chemical Processes in Lakes* (Wiley, New York, 1985).
10. Trefry, J. H., Metz, S., Trocine, R. P. & Nelson, T. A. *Science* **230**, 439–441 (1985).
11. Honeyman, B. D. & Santschi, P. *Envir. Sci. Technol.* **22**, 862 (1988).
12. Munawar, M. & Munawar, I. F. *Hydrobiologia* **247**, 181 (1986).
13. Boyle, E. A., Chapnick, S., Shen, G. T. & M. P. Bacon, M. P. *J. geophys. Res.* **91**, 8573–8593 (1986).
14. *Lead in the Canadian Environment: Science and Regulation*, Final Report of the Commission of Lead in the Environment, Royal Society of Canada, Ottawa (1986).

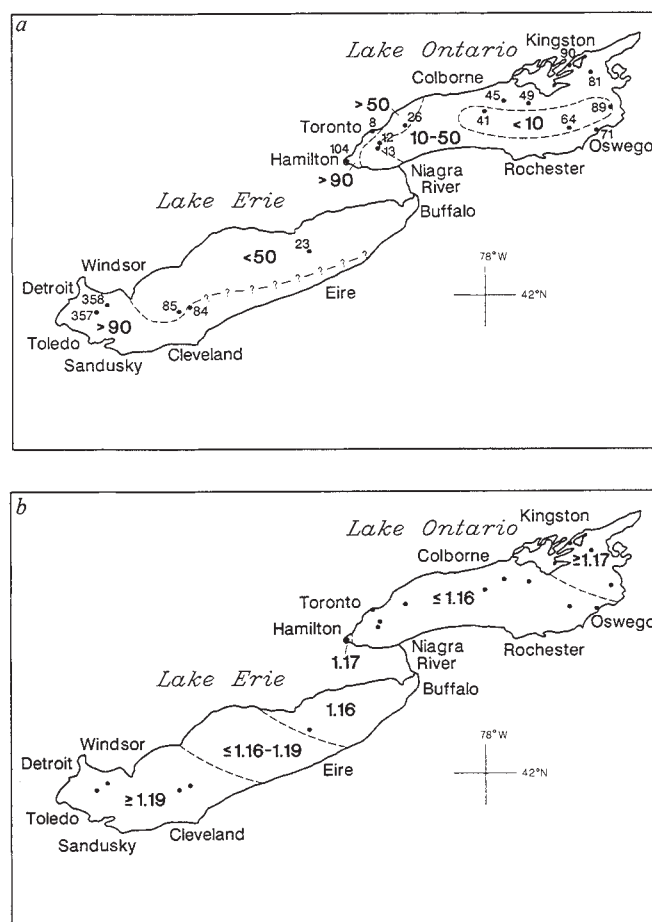


FIG. 1 Distributions of a, dissolved ($\leq 0.4 \mu\text{m}$) lead concentrations (pmol kg^{-1}) and b, isotopic compositions ($^{206}\text{Pb}/^{207}\text{Pb}$) in surface (1 m) waters of Lake Ontario and Lake Erie (August 1987). The station numbers, corresponding to those in Table 1, are indicated.

15. *Air Quality Criteria for Lead*, U.S. Environmental Protection Agency, Triangle Park (1986).
16. Stukas, V. J. & Wong, C. S. *Science* **211**, 1424–1427 (1981).
17. Flegal, A. R., Rosman, K. J. & Stephenson, M. R. *Envir. Sci. Technol.* **21**, 1075–1079 (1987).
18. Schindler, D. W. *Science* **239**, 149–157 (1988).
19. Maring, H., Settle, D. M., Buat-Menard, P., Dulac, F. & Patterson, C. C. *Nature* **330**, 154–156 (1987).

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High gradients of lead isotopic composition in north-east Pacific upwelling filaments

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SUBSTANTIAL differences in stable lead isotopic composition in coastal waters of the north-east Pacific have been inferred from a single vertical profile taken near western Canada¹. Here we present measurements of lead isotopes in two upwelling filaments off central California (39° N, 124° W) which confirm that such marked differences exist. The distinct, filamentary structures appear in association with surface jets which transport cold upwelled water hundreds of kilometres offshore². Surface-water ratios of stable lead isotopes (^{204}Pb , ^{206}Pb , ^{207}Pb , ^{208}Pb) within the core of the first filament (for example, $^{206}\text{Pb}/^{207}\text{Pb} = 1.17$) contrasted with those of surrounding coastal waters ($^{206}\text{Pb}/^{207}\text{Pb} = 1.19$) and those of industrial lead aerosols from the United States ($^{206}\text{Pb}/^{207}\text{Pb} = 1.22$). Contrasts in the isotopic composition indicated that the lead within the core was primarily derived from aeolian inputs of Asian industrial lead ($^{206}\text{Pb}/^{207}\text{Pb} = 1.16$) to remote waters, which were then upwelled near the North American coastline. High gradients of lead isotopic composition in coastal waters were confirmed by surface and sub-surface measurements surrounding a second upwelling filament. These gradients illustrate the complexity of trace-element cycles in coastal waters, and the offshore fluxes within the ephemeral filaments demonstrate significant influences of coastal dynamics on composition within the ocean gyre.

Two-litre samples of surface water were collected from a ship-launched raft, using established procedures³, along transects across an upwelling filament (F1) with a jet-like offshore flow of 0.5 m s^{-1} during the OPTOMA-21 cruise off central California, 7–20 July 1986 (Table 1a). Sub-surface samples were then collected with modified Go-Flo devices⁴ along transects across a second filament (F2) during a return cruise (ONR-2), 22–29 July 1986 (Table 1b). Sampling locations are shown in Fig. 1, with the thermal boundaries of the filaments determined from satellite infrared imagery of sea surface temperatures. Lead concentrations and isotopic compositions were measured by thermal ionization mass spectrometry with established procedures at Caltech³, and intercalibrated with replicate analyses at Lawrence Livermore National Laboratory.

Isotopic compositions of lead ($1.170 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.173$) within the core of F1 (stations 64, 171, and 174) were similar to those of north-east Pacific sub-arctic surface waters ($1.163 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.175$). The latter are primarily due to atmospheric deposition of Asian industrial lead aerosols ($^{206}\text{Pb}/^{207}\text{Pb} = 1.163$) advected across the North Pacific by the prevailing westerly winds^{1,3}. The lead in the filament was predominantly Asian industrial lead, assuming that it was composed only of lead having $^{206}\text{Pb}/^{207}\text{Pb}$ ratios characteristic of Asian and US industrial sources. This assumption was confirmed by the highly

significant ($p < 0.01$) correlation coefficient ($R = 0.972$) of the simple linear regression of $^{206}\text{Pb}/^{207}\text{Pb}$ and $^{206}\text{Pb}/^{208}\text{Pb}$ ratios of all of the F1 samples. Lead isotope ratios of F2 fit the same regression ($p < 0.01$, $R = 0.946$).

The low salinity of the upwelled water (< 33.2 practical salinity units (p.s.u.)) indicated that it was sub-arctic water carried equatorward by the California Current. Climate data⁵ show that the upwelled water did not originate more than 200 km directly offshore of the filament and did not represent cross-shore mass exchange over scale lengths reaching the offshore transition zone⁶, because mean salinities and mean temperatures in isopycnals which outcrop near the coast change significantly along cross-shore transects.

The remote origin of lead in F1 was substantiated by the contrasting isotopic compositions ($^{206}\text{Pb}/^{207}\text{Pb} = 1.190 \pm 0.001$) of surrounding surface waters (stations 167 and 176, Fig. 1). The isotopic composition of lead in surrounding waters were between those of industrial leads from Asia and the United States, and were consistent with previous measurements of lead isotopic composition in temperate surface waters in the north-east Pacific^{1,3}. Ratios of inshore surface waters ($1.175 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.184$ at stations 15, 48, 183, 188) were between those of the upwelling filament and offshore ($\sim 100 \text{ km}$) surface waters, suggesting mixture of upwelled and ambient surface water.

The pronounced differences in lead isotopic compositions within north-east Pacific coastal surface waters contrasted with their relative homogeneity within surface waters of the North Pacific gyre³. The coastal inhomogeneity is attributed to the relative stability of the jets at scale lengths greater than tens of kilometres, although shear-flow instabilities at wavelengths of $\sim 20 \text{ km}$ have been observed⁷. Although similarly large gradients in lead isotopic compositions have been observed near point sources in Puget Sound⁸ and Monterey Bay⁹, they were found to be rapidly eroded by turbulent mixing.

Local circulation patterns influenced the isotopic composition of surface waters surrounding F1. A lower proportion of US industrial lead appeared in the surface water of the cyclonic shear zone south of the filament where the thermocline was elevated, and a higher proportion was found where the thermocline was depressed. This may reflect the exposure time to atmospheric

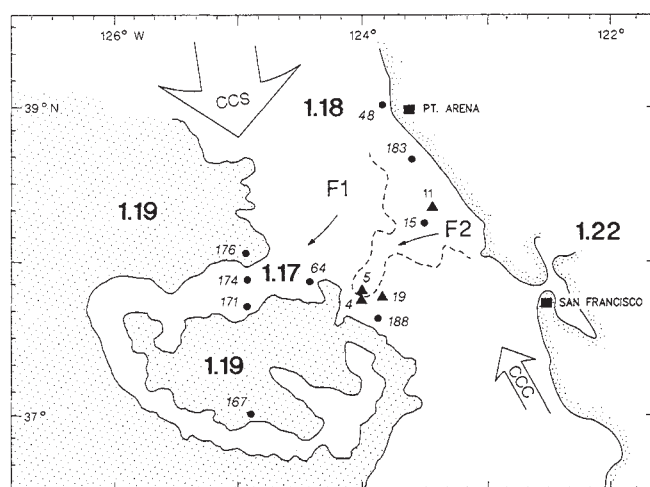


FIG. 1 Sampling locations of OPTOMA-21 (●) and ONR-86 (▲) cruises on 7–20 July 1986 and 22–29 July 1986, respectively. Positions of two sequentially formed upwelling filaments (F1 and F2) are based on satellite infrared images of sea surface temperatures. Offshore flows of F1 and F2 and longshore flows of the California current system (CCS) and the counter-current (CCC) are indicated by arrows. $^{206}\text{Pb}/^{207}\text{Pb}$ ratios of US industrial lead aerosols (1.22) and surface waters offshore (1.19), inshore (1.18) and within the core of the F1 upwelling filament (1.170) are also shown.

sources of US industrial lead. Dynamic height contours imply a poleward flow along the coastline, a feature more commonly observed during the winter¹⁰. Upwelled water entrained into F2 near the coast may have contained enhanced US industrial lead as a result of the poleward flow, compared to water upwelled and entrained further from the coast, under the hypothesis of close proximity of the near-shore flow to atmospheric and fluvial inputs of industrial lead. The slightly warmer temperatures of the coastal (shelf) source water relative to the offshore source water suggests that the shelf water upwelled from a shallower depth, with greater likelihood of exposure to US industrial lead. Poleward flow and uptake of US industrial lead, along with vertical migration, has also been suggested to explain variable lead isotopic composition near western Canada¹.

The predominantly Asian origin of lead in the surface waters of the F1 filament was corroborated by vertical profiles of lead isotopic compositions in the F2 filament. The low $^{206}\text{Pb}/^{207}\text{Pb}$ ratios (1.173–1.180) of sub-surface waters (50 to 150 m depth) in the core of F2 (station 5) indicated that >80% of its lead was derived from Asian industrial lead aerosols (Fig. 2). The isotopic composition of the near-surface waters surrounding F2 were consistent with those surrounding F1. Cool water inshore of F2 (station 11) had $^{206}\text{Pb}/^{207}\text{Pb} = 1.181$, within the range of the waters inshore of the F1 filament ($1.174 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.184$).

However, surface water within the F2 core (station 5) contained an anomalously high percentage of US industrial lead ($^{206}\text{Pb}/^{207}\text{Pb} = 1.204$). More than one-half of its lead was from recent inputs of US industrial lead, under the assumption that the lead in that sample was a mixture of US industrial lead and lead from the offshore surface waters. This was consistent with ^{210}Pb measurements which suggested a local origin of lead in

the filament¹¹. The offshore transport of US industrial lead in F2 was still less than that in F1, as the volume transport of F1 ($1.5 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ or 1.5 Sv)¹² was about six times than that of the second filament (0.25 Sv)¹³.

Jets associated with upwelling filaments can advect a cool, narrow stream of water hundreds of kilometres offshore, with a typical velocity of 0.5 m s^{-1} , a temperature anomaly of 2°C and a width of 30–40 km^{2,7}. The surface offshore transport during periods of numerous simultaneous jets can exceed the longshore California current system (CCS) transport, as cross-shore transport per jet commonly exceeds 2 Sv, which is 20% of the CCS climatological mean equatorial transport. This produces cross-shore exchange of water, heat, nutrients, biota and pollutants². Cross-shore gradients of climatological data show that jets are fed from longshore coastal flows, and that offshore transport and dispersal can result for constituents with coastal sources and short timescales, such as US lead (surface-water residence time of ≤ 2 years³).

The offshore lead flux within F1 can be estimated. Lead concentrations within the jet (peak velocity 0.55 m s^{-1} , volume transport 1.5 Sv) were $\sim 50 \text{ pmol kg}^{-1}$ (Table 1a). The ephemeral offshore transport of lead in the jet associated with F1 ($1.7 \times 10^{10} \text{ ng s}^{-1}$ over 250 km) was $\sim 20\%$ of the expected longshore transport of lead in the CCS. The horizontal flux of lead at the surface of the narrow filament (taken to be 40 km wide and 50 m deep) was six orders of magnitude greater than the flux of lead aerosols to those waters, based on the annual aeolian flux of industrial lead to temperate surface waters in the North Pacific ($15 \text{ ng cm}^2 \text{ yr}^{-1}$)¹⁴. It was also seven orders of magnitude greater than the concurrent flux of natural lead to those waters, based on Pleistocene sediment deposition rates in the North Pacific

TABLE 1 Lead concentrations and isotopic compositions in the north-east Pacific associated with upwelling filaments F1 and F2

(a) Surface-water samples collected in transects across upwelling filament F1 on the OPTOMA-21 cruise

Station no.	Lat. ($^\circ\text{N}$); Long. ($^\circ\text{W}$)	Pb (pmol kg^{-1})	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{206}\text{Pb}/^{207}\text{Pb}$	$^{206}\text{Pb}/^{208}\text{Pb}$
15	38°14'; 123°30'	21.7	18.152 $\pm$ 0.006	1.1746 $\pm$ 0.0009	0.4825 $\pm$ 0.0011
48	39°00'; 123°48'	25.3	18.261 $\pm$ 0.004	1.1804 $\pm$ 0.0008	0.4841 $\pm$ 0.0022
64	37°51'; 124°25'	12.5	—	1.1655 $\pm$ 0.0024	0.4793 $\pm$ 0.0034
167	37°02'; 124°55'	7.4	—	1.1932 $\pm$ 0.0051	0.4910 $\pm$ 0.0099
171	37°51'; 124°56'	10.6	18.158 $\pm$ 0.003	1.1750 $\pm$ 0.0013	0.4833 $\pm$ 0.0035
174	37°49'; 124°57'	11.5	18.322 $\pm$ 0.008	1.1711 $\pm$ 0.0028	0.4821 $\pm$ 0.0039
176	38°02'; 124°57'	15.0	18.253 $\pm$ 0.013	1.1869 $\pm$ 0.0047	0.4882 $\pm$ 0.0067
183	38°29'; 123°33'	18.5	—	1.1845 $\pm$ 0.0013	0.4864 $\pm$ 0.0016
188	36°37'; 123°49'	18.2	18.177 $\pm$ 0.003	1.1745 $\pm$ 0.0018	0.4809 $\pm$ 0.0018

Standard deviation of lead concentration measurements is $\pm 5\%$.

(b) Sub-surface waters collected in vertical profiles in and adjacent to upwelling filament F2 on the ONR-86 cruise*

Station no.	Lat. ($^\circ\text{N}$); Long. ($^\circ\text{W}$)	Depth (m)	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{206}\text{Pb}/^{207}\text{Pb}$	$^{206}\text{Pb}/^{208}\text{Pb}$
4	37°42'; 124°06'	10	18.184 $\pm$ 0.008	1.1877 $\pm$ 0.0018	0.4868 $\pm$ 0.0025
		50	18.320 $\pm$ 0.013	1.1742 $\pm$ 0.0003	0.4802 $\pm$ 0.0013
		75	18.199 $\pm$ 0.020	1.1546 $\pm$ 0.0006	0.4760 $\pm$ 0.0004
		100	18.250 $\pm$ 0.013	1.1695 $\pm$ 0.0003	0.4785 $\pm$ 0.0009
		150	18.155 $\pm$ 0.003	1.1632 $\pm$ 0.0010	0.4761 $\pm$ 0.0015
5	37°46'; 124°00'	10	—	1.2036 $\pm$ 0.0023	0.4929 $\pm$ 0.0013
		50	—	1.1800 $\pm$ 0.0020	0.4861 $\pm$ 0.0040
		75	18.362 $\pm$ 0.002	1.1784 $\pm$ 0.0007	0.4822 $\pm$ 0.0003
		100	18.339 $\pm$ 0.008	1.1744 $\pm$ 0.0013	0.4809 $\pm$ 0.0015
		150	18.129 $\pm$ 0.020	1.1684 $\pm$ 0.0009	0.4799 $\pm$ 0.0010
11	38°21'; 123°27'	10	—	1.1808 $\pm$ 0.0066	0.4847 $\pm$ 0.0084
		50	18.399 $\pm$ 0.001	1.1799 $\pm$ 0.0004	0.4812 $\pm$ 0.0001
		75	18.380 $\pm$ 0.018	1.1804 $\pm$ 0.0006	0.4823 $\pm$ 0.0005
		135	18.402 $\pm$ 0.018	1.1800 $\pm$ 0.0004	0.4814 $\pm$ 0.0002
		100	—	—	—
19	37°44'; 123°51'	10	—	—	—
		50	—	1.1903 $\pm$ 0.0059	0.4892 $\pm$ 0.0036
		75	—	1.1696 $\pm$ 0.0031	0.4807 $\pm$ 0.0015
		100	18.144 $\pm$ 0.023	1.1643 $\pm$ 0.0006	0.4758 $\pm$ 0.0004

* ^{210}Pb activities and trace-element concentrations reported by Shiller *et al.*¹¹.

Standard deviation of lead concentration measurements is $\pm 5\%$. The isotopic composition of lead is $x \pm 95\%$ confidence limit.

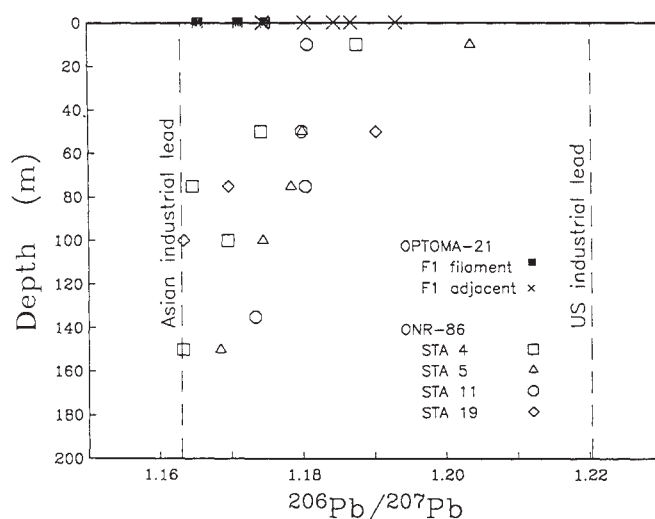


FIG. 2 $^{206}\text{Pb}/^{207}\text{Pb}$ ratios of coastal waters off central California during the July 1986 upwelling regimes. Surface waters were collected within the core of upwelling filament F1 (●) and adjacent waters (x) on the OPTOMA-21 cruise 7–20 July 1986. Sub-surface waters were collected in the core of upwelling filament F2 (station 5) and adjacent locations (stations 4, 11 and 19) on the ONR-2 cruise 20–29 July 1986. Contributions of industrial leads from Asia and the United States are indicated by the relative proximities of seawater $^{206}\text{Pb}/^{207}\text{Pb}$ ratios to those of industrial lead aerosols from the two sources.

($3 \text{ ng cm}^{-2} \text{ yr}^{-1}$)³, showing the strong influence of horizontal advection on vertical profiles of isotopic composition. While the net offshore transport of lead appears to be limited by relatively small differences of mean lead concentrations in the westward jet and return flow waters, a net offshore transport of US lead may exist in filament structures, implying rapid offshore transport of coastally derived constituents.

The Asian lead signature of sub-surface water and filament core water suggests a longshore and then offshore route of the upwelled filament water, because of the average cross-shore salinity gradients along isopycnals⁵. Baroclinic instability of the longshore currents may explain the generation of eddies, meanders and jets¹⁵, with upwelling enabling the cold filament surface expression. Ambient offshore water and lead must mix horizontally with filaments along their courses, with resultant partial homogenization of lead concentrations and isotopic compositions. Therefore, coastal mixing and offshore transport of lead associated with upwelling filaments must contribute to the relative homogeneity of vertical lead profiles in the north-east Pacific. □

Influence of atmospheric pollution on nutrient limitation in the ocean

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IN the midst of the debate over the ocean being phosphorus- or nitrogen-limited^{1,2}, the 'acid rain' controversy prompted studies related to the atmospheric transport and delivery of pollutant nitrogen compounds over the ocean^{3–9}. Some of those investigations concluded that atmospheric nitrogen had only minimal effects on euphotic-zone productivity^{7,8} or on nitrate at the Atlantic thermocline⁹, thus suggesting a negligible oceanic role for pollutant atmospheric nitrogen. Here I give evidence to the contrary, by clearly showing that, whereas nitrogen limitation is much more prevalent than phosphorus limitation in the surface ocean, those areas with the strongest indications of phosphorus limitation in the North Atlantic and North Pacific are downwind of the most populated and urbanized regions of eastern Asia and North America. This geographic coincidence and the timing and composition of atmospheric nitrogen deposition suggest a plausible, albeit untested, mechanism whereby airborne pollutant nitrogen can lead to phosphorus limitation.

The principal evidence comes from officially released computer tapes of two large, comparable oceanic data sets: (1) GEOSECS data for all three major oceans between 1972 and 1978^{10–12}, and (2) 'Transient Tracers in the Ocean' (TTO) data for the North and Tropical Atlantic in 1981–1983^{13,14}. Although most of the expeditions' samplings were below the euphotic zone, enough nutrient data were taken within the zone that its large-scale patterns of depletion of nutrient nitrogen (nitrate + nitrite) and phosphorus (phosphate) could be discerned. The underlying rationale was that the nutrient more likely to be limiting to primary production should show a greater frequency of very low (that is, undetectable or zero) concentrations in the oligotrophic euphotic zone. In a total of 816 GEOSECS and TTO water samples, at least one of these nutrients was reported to have a concentration that was indistinguishable from zero. The samples were obtained at 365 stations worldwide (Table 1).

Coherence of the patterns of nutrient undetectability depends on the internal consistency and comparability of the data sets. In both respects the data were excellent because the same techniques were used by GEOSECS and TTO analysts under the auspices of the Physical and Chemical Oceanographic Data Facility at Scripps Institution of Oceanography^{15,16}. Detection limits exhibited consistent variabilities, as can be seen in estimates derived from the application of Student's *t*-distribution ($P=0.05$) to duplicate analyses within the data sets. When expressed as a mean ± 2 standard deviations, limits based on GEOSECS duplicates were $0.018 \pm 0.006 \mu\text{mol kg}^{-1}$ for phosphate, $0.003 \pm 0.010 \mu\text{mol kg}^{-1}$ for nitrite and $0.17 \pm 0.11 \mu\text{mol kg}^{-1}$ for nitrate. From TTO duplicates, limits were $0.015 \pm 0.001 \mu\text{mol kg}^{-1}$ for phosphate, $0.008 \pm 0.004 \mu\text{mol kg}^{-1}$ for nitrite and $0.29 \pm 0.14 \mu\text{mol kg}^{-1}$ for nitrate. The standard deviations indicate that the mean detection limits for the respective methods remained statistically unchanged despite the 5 to 10 years separating the GEOSECS and TTO expeditions. In fact, purely analytical detection limits should have been even closer because GEOSECS and TTO duplicate samples were taken from different bottles tripped at the same depths on the same casts.

A correction was required because nitrite concentrations were not reported for some of the GEOSECS samples. The unreported nitrite concentrations were assumed to be zero. For samples with detectable (that is, non-zero) nitrate concentrations, this

Received 21 February; accepted 24 April 1989.

1. Flegal, A. R., Itoh, K., Patterson, C. C. & Wong, C. S. *Nature* **321**, 689–690 (1986).
2. Moores, C. N. K. & Robinson, A. R. *Science* **223**, 51–53 (1984).
3. Flegal, A. R. & Patterson, C. C. *Earth planet. Sci. Lett.* **64**, 19–32 (1983).
4. Martin, J. H., Knauer, G. A. & Broenkow, W. W. *Deep Sea Res.* **32**, 2307–2322 (1985).
5. Lynn, R. J., Bliss, K. A. & Eber, L. E. *CalCOFI Atlas No. 30* (1982).
6. Roden, G. I. *J. geophys. Res.* **76**, 3462–3474 (1971).
7. Washburn, L. & Armi, L. J. *J. phys. Oceanogr.* **18**, 1075–1092 (1988).
8. Stukas, V. J. & Wong, C. S. *Science* **211**, 1424–1427 (1981).
9. Flegal, A. R., Rosman, K. J. & Stephenson, M. R. *Envir. Sci. Tech.* **21**, 1705–1079 (1987).
10. Hickey, B. M. *Prog. Oceanogr.* **8**, 191–279 (1979).
11. Shiller, A. M., Broenkow, W. W., Elrod, V. A. & Martin, J. H. *Eos* **67**, 1047 (1986).
12. Bucklin, A., Rienecker, M. M. & Moores, C. N. K. *J. geophys. Res.* (in the press).
13. Elrod, V. A. thesis, Moss Landing Mar. Lab. (1987).
14. Maring, H., Patterson, C. C. & Settle, D. in *Chemical Oceanography* (Academic, London, in the press).
15. Batteen, M. L. in *Poleward Flows Along Eastern Ocean Boundaries* (Springer, Berlin, in the press).

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assumption clearly does not influence whether or not the sum of nitrate and nitrite was zero. For the following reasons, the assumption was also applied to samples with undetectable nitrate concentrations. Zero-nitrate GEOSECS samples from the Atlantic were of greatest concern, because nitrite concentrations were reported for only 29% of them. However, in 94% of this 29%, nitrite was zero. In addition, more than 99% of the zero-nitrate TTO Atlantic samples had reported nitrite concentrations, and 83% of those were zero. GEOSECS data from the Pacific and Indian oceans were less problematic because the percentages of their zero-nitrate samples with reported nitrite concentrations were 93% and 100% respectively.

A correction was also required for the phosphate concentrations because these included a contribution from arsenate, which combines with molybdate to form a heteropolyacid with a molar absorptivity equal to that of the phosphomolybdate complex in the standard analytical phosphate technique¹⁷. In the upper 200 m, arsenate levels were 0.015–0.024 $\mu\text{mol kg}^{-1}$ in the North-east Pacific¹⁸, 0.018–0.023 $\mu\text{mol kg}^{-1}$ in the Cape Basin¹⁹ and $0.028 \pm 0.010 \mu\text{mol kg}^{-1}$ in the North Atlantic²⁰. The North Atlantic data²⁰ have a higher degree of uncertainty than the other data because a less sensitive spectrophotometric method

was used. On the basis of these data, the typical arsenate concentration in the euphotic zone was therefore taken to be 0.02 $\mu\text{mol kg}^{-1}$. Phosphate concentrations of 0.02 $\mu\text{mol kg}^{-1}$ or less were set equal to zero, and all other reported phosphate concentrations were reduced by 0.02 $\mu\text{mol kg}^{-1}$ as a correction.

After the two corrections, GEOSECS and TTO samples with zero nutrient concentrations were classified into three categories:

- (1) $[\text{NO}_3^- + \text{NO}_2^-] = 0, [\text{PO}_4^{3-}] > 0$
- (2) $[\text{NO}_3^- + \text{NO}_2^-] > 0, [\text{PO}_4^{3-}] = 0$
- (3) $[\text{NO}_3^- + \text{NO}_2^-] = 0, [\text{PO}_4^{3-}] = 0$

The classified samples were examined for frequency distribution among the categories and for geographical variations (Table 1 and Fig. 1). Most of the samples came from depths of 50 m or less, although a few came from depths of 150–160 m.

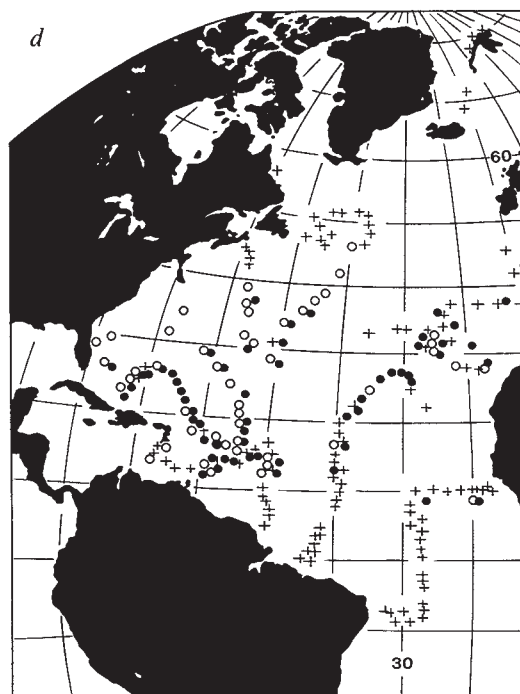
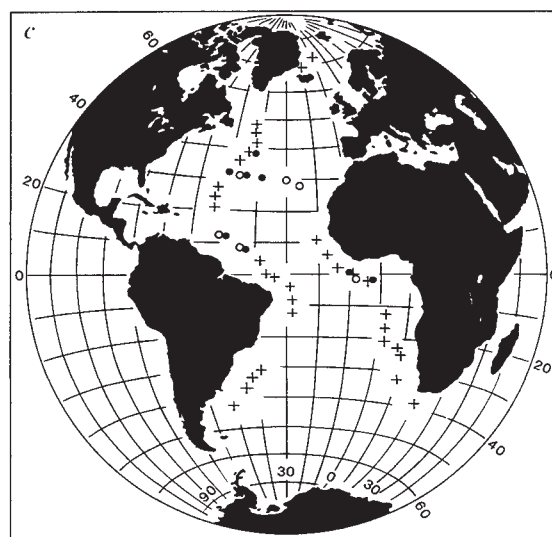
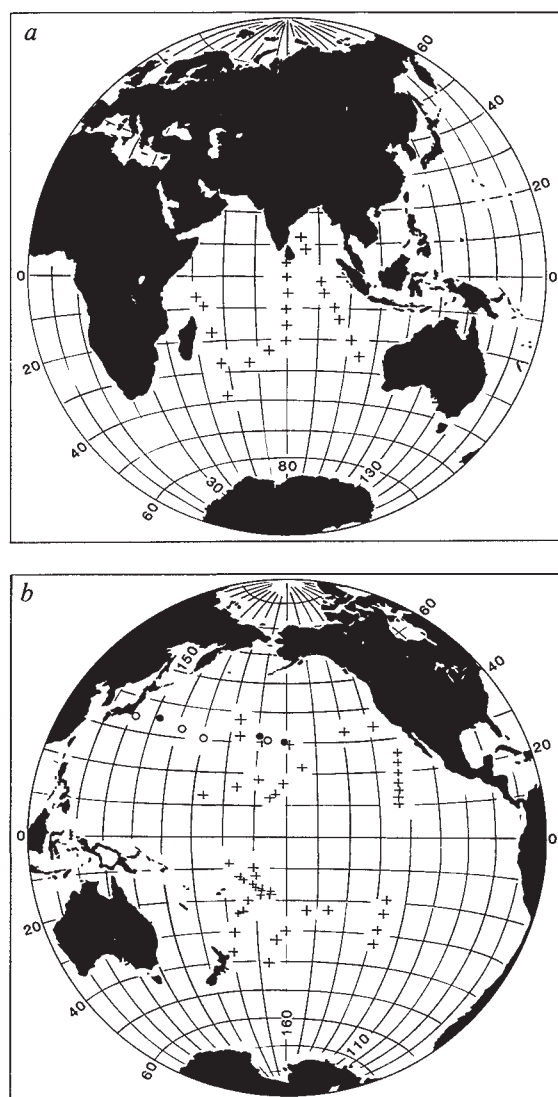


FIG. 1 Distribution of the detectability of the nutrients nitrate+nitrite and phosphate in the upper 200 m of the world oceans. + denotes samples with undetectable nitrate+nitrite and detectable phosphate; o denotes samples with undetectable phosphate and detectable nitrate+nitrite; and ● denotes samples with undetectable nitrate+nitrite and phosphate. a, Indian Ocean, GEOSECS data, 1977–1978¹². b, Pacific Ocean, GEOSECS data, 1973–1974¹¹. c, Atlantic Ocean, GEOSECS data, 1972–1973¹⁰. d, Atlantic Ocean, TTO data, North Atlantic (TTO/NAS) in 1981¹³, and Tropical Atlantic (TTO/TAS) in 1982–1983¹³.

Indian Ocean, GEOSECS data, 1977–1978¹². b, Pacific Ocean, GEOSECS data, 1973–1974¹¹. c, Atlantic Ocean, GEOSECS data, 1972–1973¹⁰. d, Atlantic Ocean, TTO data, North Atlantic (TTO/NAS) in 1981¹³, and Tropical Atlantic (TTO/TAS) in 1982–1983¹³.

Table 1 clearly shows the predominance of nitrogen limitation as defined by nutrient detectability. Of the 816 samples with one or both nutrients missing, 68% had undetectable nitrate + nitrite and detectable phosphate (that is, in category 1). In contrast, the reverse situation of phosphorus limitation (category 2) was observed in only 14% of the samples, and both nutrients were reported missing in 18% of the samples (category 3). The strength of the nitrogen limitation is further substantiated by the fact that an average of 50% of the samples in category 1 had phosphate concentrations of between 5 and 24 times the phosphate detection limit, whereas none of the category 2 samples had nitrate + nitrite concentrations that even reached 5 times the nitrate + nitrite detection limit; their highest nitrate + nitrite value was only 2.3 times this limit. Thus even the phosphorus-limited samples tended to be close to nitrogen limitation, whereas at least half of the nitrogen-limited samples were well beyond phosphorus limitation. For these calculations, detection limits were considered to be $0.02 \mu\text{mol kg}^{-1}$ for phosphate and $0.24 \mu\text{mol kg}^{-1}$ for nitrate + nitrite (see above).

Some of the TTO data in Table 1 do not closely follow the trend of the combined data from all sources. Nitrogen limitation for TTO North Atlantic data was only slightly more frequent than phosphorus limitation (see discussion below).

Samples in the three categories of nutrient undetectability are not distributed randomly but tend to be clumped in discrete zones (Fig. 1a-d). This finding, along with the fact that the samples are not distributed equally among these categories in Table 1, argues against the possibility that ordinary random analytical variability is responsible for a sample falling into one category rather than another. Nitrogen-deficient samples are the only type found in the oligotrophic Indian ocean, in most of the oligotrophic Pacific and in the mid-latitude waters of the South Atlantic. Samples with phosphate absent are found almost exclusively in the central North Atlantic and the western North Pacific. The high sampling density of the TTO North Atlantic study in the region where phosphorus limitation seems to be important thus accounts for the weak dominance of nitrogen-depleted samples in the data for that study in Table 1.

The distribution of categories 2 and 3 in Fig. 1 is particularly intriguing in view of recent work by Levy and Moxim on the production, concentration and deposition of combustion-produced atmospheric nitrogen²¹⁻²³. Their global map²² of the atmospheric concentration of surface-layer anthropogenic nitrogen at 990 mbar pressure shows that eastern North America and the western North Atlantic from Newfoundland to Cuba have very high levels of this nitrogen: 1 part per 10^9 by volume (p.p.b.v.) to over 10 p.p.b.v. Most of the atmosphere over the oceans has levels of <0.005 – 0.1 p.p.b.v. N. Their map also shows another region of high average concentration of >1 p.p.b.v. N over eastern Asia from northern Vietnam to Sakhalin island, including all of the Japanese archipelago with its urbanized industrial centres. No comparable areas of high combusted-nitrogen levels are reported anywhere around the Indian ocean.

Oceanic impacts are predicted by their measurement-tested models of wet²² and total²³ atmospheric fossil-fuel nitrogen deposition. Total fossil-fuel deposition rates are 1 – $10 \text{ mmol N m}^{-2} \text{ yr}^{-1}$ for the Atlantic between 25°N and North America, 1 – $10 \text{ mmol N m}^{-2} \text{ yr}^{-1}$ for the half of the northwestern Pacific that is adjacent to Asia, and less than $1 \text{ mmol N m}^{-2} \text{ yr}^{-1}$ (mostly 0.01 – $0.1 \text{ mmol N m}^{-2} \text{ yr}^{-1}$) for the rest of the ocean.

Wherever high levels of pollutant atmosphere nitrogen occur over the continents, there is an increase in the downwind incidence of undetectable phosphate in the neighbouring surface ocean ($\circ$ and $\bullet$ in Fig. 1b-d). In the absence of high anthropogenic nitrogen over the continents, nutrient-nitrogen depletion seems to predominate, as in the central Indian Ocean (+ in Fig. 1a).

The following mechanism may be at work. Prevailing wind directions and storm tracks^{4,6,24} are consistent with the transport and deposition of fixed nitrogen products from eastern Asia and North America over the western North Pacific and Atlantic, respectively. This deposition no doubt supplies nutrient nitrogen to the sea surface but probably does not materially enhance euphotic-zone productivity in either region⁸. For example, new production in the Sargasso Sea should require 220 – $1,800 \mu\text{mol N m}^{-2} \text{ d}^{-1}$, whereas atmospheric deposition provides no more than $128 \mu\text{mol N m}^{-2} \text{ d}^{-1}$ and probably less than $50 \mu\text{mol N m}^{-2} \text{ d}^{-1}$ (refs 7, 8). However, if the growth of phytoplankton populations in the two regions has consumed most of the available phosphate, the injection of fixed atmospheric nitrogen might be just large enough to stimulate additional growth that will reduce the nearly depleted phosphate to undetectable levels. Nitrate and nitrite may be reduced to undetectability at the same time.

There are several factors to be considered in evaluating this proposed mechanism. The argument for it is based primarily on the proximity of areas of apparent phosphorus limitation to regions with high levels of atmospheric nitrogen oxides. The steps of the mechanism have yet to be identified and quantitatively estimated in coordinated meteorological, hydrographic and biological studies. More winter-time, surface-ocean nutrient data than are found in the GEOSECS and TTO sets would be desirable for comparison with the atmospheric-deposition data. There is, however, important supporting evidence, especially in the North Atlantic. In addition to the favourable wind patterns and storm tracks off the continents, high nitrogen/phosphorus ratios (320 – $1,640 \text{ mol N/mol P}$ over the Sargasso Sea and 670 – $5,200 \text{ mol N/mol P}$ over the North Pacific⁸) in atmospheric deposition are consistent with the mechanism. In the Sargasso Sea, maximum atmospheric nitrogen input occurs in winter when the greatest number of storm fronts reach the open Atlantic^{3,4}. In contrast, although the maximum upward mixing of deeper waters to the euphotic zone also occurs in winter, the largest nitrate/phosphate ratios in deeper waters are only 104 mol N/mol P for the Sargasso Sea and 17 mol N/mol P for the North Pacific, according to GEOSECS and TTO data. Based

TABLE 1 Numbers and geographic distributions of oligotrophic GEOSECS and TTO seawater samples having undetectable nutrients.

Data source	Stations	Samples	Nutrients absent		
			$\text{NO}_3 + \text{NO}_2$ (Category 1)	PO_4 (Category 2)	Both (Category 3)
GEOSECS					
Atlantic	46	121	90 (29)	10 (0)	21
Pacific	56	149	126 (95)	9 (0)	14
Indian	22	53	53 (31)	0 (0)	0
TTO					
North Atlantic	135	252	90 (26)	82 (0)	80
Tropical Atlantic	106	241	199 (96)	11 (0)	31
Total	365	816	558(277)	112 (0)	146

The values in parentheses indicate the numbers of samples in categories 1 or 2 which had the detected nutrient present in a concentration at least 5 times its detection limit. See text for the explanation of the categories and detection limits.

on these ratios, the atmospheric source therefore has a much greater potential for forcing oligotrophic phytoplankton toward phosphorus limitation.

Closer to the margins of North America and Asia, interactions of surface waters with, respectively, the Gulf Stream and Kuroshio currents may alter the proposed mechanism, as eddies are shed and decay. However, the data in Fig. 1b-d show that most of the phosphorus limitation occurs in the central subtropical gyres of the North Atlantic and Pacific, far away from those currents.

The proposed mechanism provides a plausible explanation for the fact that the two areas of the surface ocean with the greatest tendency toward phosphorus limitation lie adjacent to the highest concentrations of combusted atmospheric nitrogen. This suggests that a future reduction in the airborne products of human industrial activity might permit those two areas to shift toward the nitrogen-limited condition that appears to dominate in the rest of the oligotrophic eutrophic zone. □

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1. Doremus, C. *Biol. Oceanogr.* **1**, 429-436 (1982).
2. Smith, S. V. *Limnol. Oceanogr.* **29**, 1149-1160 (1984).

3. Church, T. M., Galloway, J. N., Jickells, T. D. & Knap, A. H. *J. geophys. Res.* **87**, 11013-11018 (1982).
4. Jickells, T., Knap, A. H., Church, T., Galloway, J. & Miller, J. *Nature* **297**, 55-57 (1982).
5. Paerl, H. *Nature* **316**, 747-749 (1985).
6. Galloway, J. N. & Whelpdale, D. M. *Global biogeochem. Cycles* **1**, 261-281 (1987).
7. Knap, A., Jickells, T., Psenzeny, A. & Galloway, J. *Nature* **320**, 158-160 (1986).
8. Duce, R. A. in *The Role of Air-Sea Exchange in Geochemical Cycling* (ed. Buat-Menard, P.) 497-529 (Reidel, Dordrecht, 1986).
9. Peng, T. H. & Broecker, W. S. *J. geophys. Res.* **89**, 8170-8180 (1984).
10. Bainbridge, A. E. *GEOSCEAS Atlantic Expedition 1*, Hydrographic Data 1972-1973 (US Government Printing Office, 1981).
11. Broecker, W. S., Spencer, D. W. & Craig, H. *GEOSCEAS Pacific Expedition 3*, Hydrographic Data 1973-1974 (US Government Printing Office, 1982).
12. Weiss, R. F., Broecker, W. S., Craig, H. & Spencer, D. W. *GEOSCEAS Indian Ocean Expedition 5*, Hydrographic Data 1977-1978, 48 (US Government Printing Office, 1983).
13. Brewer, P. G., Sarmiento, J. L. & Smethie, W. M. Jr *J. geophys. Res.* **90**, 6903-6905 (1985).
14. Takahashi, T., Broecker, W. S. & Langer, S. J. *J. geophys. Res.* **90**, 6907-6924 (1985).
15. Atlas, E. L., Hager, S. W., Gordon, L. I. & Park, P. K. *A Practical Manual for Use of the Technicon Autoanalyzer in Seawater Nutrient Analyses* Oregon State University ref. No. 71-22 (1971).
16. Hager, S. W., Atlas, E. L., Gordon, L. I., Mantyla, A. W. & Park, P. K. *Limnol. Oceanogr.* **17**, 931-937 (1972).
17. Johnson, D. L. & Pilson, M. E. Q. *Analytica chim. Acta* **58**, 289-299 (1972).
18. Andreae, M. O. *Limnol. Oceanogr.* **24**, 440-452 (1979).
19. Statham, P. J., Burton, J. D. & Maher, W. A. *Deep-Sea Res.* **34**, 1353-1359 (1987).
20. Johnson, D. L. & Pilson, M. E. Q. *J. mar. Res.* **30**, 140-149 (1972).
21. Levy, H. II & Moxim, W. J. *Nature* **328**, 414-416 (1987).
22. Levy, H. II & Moxim, W. J. *Tellus* **41**, 256-271 (1989).
23. Levy, H. II in *Atmospheric Deposition, Proc. Baltimore Symp.* IAHS Publ 179 (in the press).
24. Duce, R. A., Unni, C. K., Ray, B. J., Prospero, J. M. & Merrill, J. T. *Science* **269**, 1522-1524 (1980).

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Extraterrestrial amino acids in Cretaceous/Tertiary boundary sediments at Stevns Klint, Denmark

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SINCE the discovery¹ nearly a decade ago that Cretaceous/Tertiary (K/T) boundary layers are greatly enriched in iridium, a rare element in the Earth's crust, there has been intense controversy on the relationship between this Ir anomaly and the massive extinction of organisms ranging from dinosaurs to marine plankton that characterizes the K/T boundary. Convincing evidence suggests that both the Ir spike and the extinction event were caused by the collision of a large bolide (>10 km in diameter) with the Earth¹⁻¹¹. Alternative explanations claim that extensive, violent volcanism¹²⁻¹⁴ can account for the Ir, and that other independent causes were responsible for the mass extinctions^{15,16}. We surmise that the collision of a massive extraterrestrial object with the Earth may have produced a unique organic chemical signature because certain meteorites, and probably comets, contain organic compounds which are either rare or non-existent on the Earth¹⁷. In contrast, no organic compounds would be expected to be associated with volcanic processes. Here we find that K/T boundary sediments at Stevns Klint, Denmark, contain both α -amino-isobutyric acid [AIB, (CH₃)₂CNH₂COOH] and racemic isovaline [ISOVAL, CH₃CH₂(CH₃)CNH₂COOH], two amino acids that are exceedingly rare on the Earth but which are major amino acids in carbonaceous chondrites^{17,18}. An extraterrestrial source is the most reasonable explanation for the presence of these amino acids.

In order for an organic cosmochemical signal to be detectable in K/T boundary sediments, the organic compounds in the bolide must be unique so that they can be differentiated from terrestrial organic molecules, and some finite fraction of these compounds must escape decomposition during the impact event and still be preserved today. It is now well established that C1 and C2 chondrites contain a vast assortment of organic compounds many of which rarely or never occur on the Earth¹⁷. It

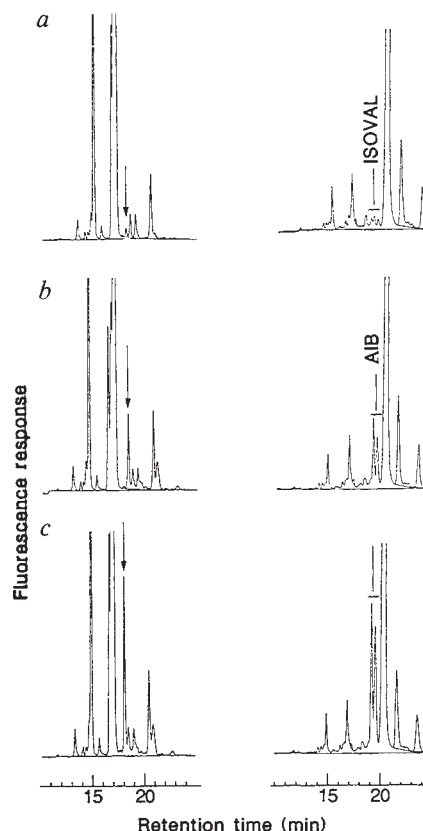


FIG. 1 OPA/NAC HPLC analyses of the AIB (left) and ISOVAL (right) fractions obtained by HCl cation-exchange chromatography²⁹ from a Stevns Klint sediment sample 0.5 m below the K/T boundary layer. a, Derivatization for 1 min; b, Derivatization for 15 min; and c, Derivatization for 15 min of extract spiked with authentic compounds. The retention times of AIB and ISOVAL are indicated by the markers. The D/L ISOVAL ratio of the standard compound was used to correct the D/L ratio measured in the sediment fraction (see Table 1). An Altex model 332 HPLC and Alltech 250 mm × 4.6 mm Econosphere C₁₈ column were used. The mobile phases were: A, 100% CH₃OH; B, 92% 50 mM NaOOCCH₃ + 8% CH₃OH titrated to pH=5.8. The column was equilibrated with 100% B. Four minutes after injection, a gradient was set up which changed to 63% B in 10 min and then to 58% in another 10 min. The flow rate was 1 ml min⁻¹. The column effluent was monitored with a Shimadzu RF-530 Fluorescence HPLC monitor at an excitation wavelength of 340 nm and an emission wavelength of 450 nm.

is not unreasonable to assume that the proposed K/T boundary impactor was this type of object because the surfaces of many asteroids are similar to carbonaceous chondrites¹⁹. Comets also likely contain a variety of organic compounds^{17,20}. Although the bulk of the organics in an impactor would probably have been pyrolysed to CO and H₂ during impact, a portion may have escaped destruction⁶ and some resynthesis may have occurred through catalytic reactions involving the released gases and the surfaces of hot mineral grains²¹.

We chose amino acids for our K/T boundary studies for the following reasons: carbonaceous chondrites contain non-protein amino acids^{17,18,22}, such as AIB and ISOVAL that are more abundant than Ir²³, are very rare in organisms²⁴, and are not known diagenetic products of terrestrial amino acids; ISOVAL stereochemistry provides an excellent indicator of the terrestrial versus extraterrestrial origin of this amino acid because it is racemic (D/L = 1.0) in meteorites²⁵ but in its rare occurrence in some fungal peptides it occurs as the D-enantiomer²³; ISOVAL has an exceedingly slow racemization rate, if it racemizes at all²⁵, and thus any biologically derived ISOVAL should still be present as the D-enantiomer even after 65 million years; and highly sensitive fluorescence-based analytical methods are available that can detect amino acids in the sub-femtomole (10^{-15} mole) range^{26,27}.

The Stevns Klint, Denmark, K/T boundary deposits were selected for our study because the boundary layer has one of the highest reported Ir concentrations^{4,28}. Eleven dried sediment samples (20–60 g) which span the Stevns Klint K/T boundary sequence were first sonicated in double-distilled water, the solids allowed to settle, and the water decanted. This process was repeated several times and was carried out to remove water-soluble or easily leached amino acids. (To minimize the possibility of contamination, only highly purified reagents were used.) The sediments were dissolved in excess 6M HCl (twice distilled) and then evaporated to dryness. The residues were hydrolysed in 6M HCl for 24 h at 100 °C and then desalted using cation-exchange chromatography. Next, these amino acid extracts were chromatographically separated using HCl cation-exchange chromatography²⁹ to obtain fractions in which AIB and ISOVAL were resolved from most other amino acids. These fractions were then analysed using *o*-phthalaldehyde/N-acetyl-L-cysteine (OPA/NAC) pre-column derivatization and high-performance liquid chromatography (HPLC)^{30,31}. With the OPA/NAC technique, AIB and ISOVAL are separated from protein amino acids and the enantiomers of ISOVAL are also resolved. We confirm³² that the yield of the fluorescent OPA derivative of α -alkyl substituted amino acids is less than that of amino acids with an α -hydrogen when derivatization is car-

ried out for 1 min at room temperature. However, the yield for AIB and ISOVAL is increased approximately 5–8 times if derivatization is carried out for 15 min at room temperature while the yield for the α -hydrogen amino acids is much less affected. We thus derivatized our mixtures for both 1 and 15 min to enhance our ability to detect AIB and ISOVAL.

The OPA/NAC HPLC results for the AIB and ISOVAL fractions for a Stevns Klint sediment sample from 0.5 m below the K/T boundary layer are shown in Fig. 1. As can be seen, peaks are clearly present that have retention times similar to those of AIB and racemic ISOVAL, and these peaks increase in size by 5–8 times with the 15-min derivatization. In addition, the peaks co-elute with the authentic compounds when they are added to the extract. In order to further demonstrate that our fractions indeed contain AIB and racemic ISOVAL, they were analysed using combined gas chromatography/mass spectrometry (GC/MS) with specific ion monitoring of the N-trifluoroacetyl-S(+)-2-butyl ester derivatives. These results are shown in Fig. 2, and they confirm the presence of both AIB and racemic ISOVAL. In order to evaluate the background levels of AIB and ISOVAL, 100 ml of 6M HCl were transferred into a clean beaker, evaporated to dryness, the residue hydrolysed, desalted and analysed in the same manner as the sediments. No AIB or ISOVAL was detected (detection limit $<10^{-12}$ moles).

The results for the samples spanning the K/T boundary at Stevns Klint are summarized in Table 1. No detectable AIB or ISOVAL is present in samples other than those within ~1 m above and below the boundary clay. A surprise is that no detectable extraterrestrial amino acid signal is found in the boundary clay itself, where the maximum Ir concentration is observed. One possible explanation is that the original extraterrestrial amino acid signal has diffused into the carbonate layers above and below the clay. Thus, although the original extraterrestrial amino acid and Ir signals were superimposed, the amino acids have subsequently been affected by diffusional and absorption processes.

What is the most likely source of the AIB and racemic ISOVAL signal in the Stevns Klint K/T boundary sediments? To determine if these amino acids could be previously undetected diagenetic products of terrestrial amino acids, we examined several other sediments and some fossils ranging in age from recent to 80 million years BP. No AIB or ISOVAL (<0.2 ng g⁻¹) was found in these samples. (These results, along with a detailed description of the general amino acid geochemistry of the Stevns Klint sediments, will be presented elsewhere.) The fact that ISOVAL is racemic provides strong evidence that its source is abiotic rather than biological. The synthesis of AIB and ISOVAL

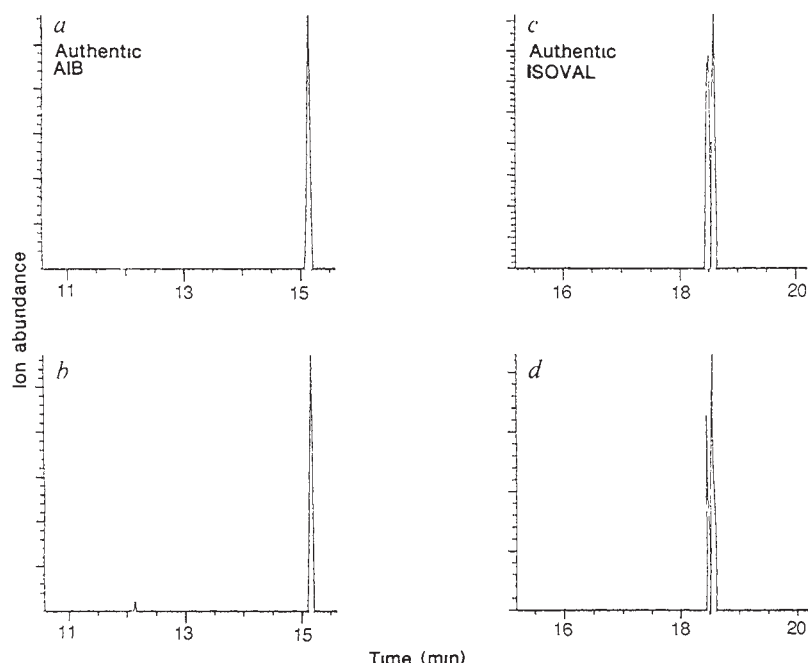
TABLE 1 Iridium and extraterrestrial amino acids in K/T boundary sediments at Stevns Klint, Denmark

Sample in relation to K/T boundary	Ir (ng g ⁻¹)	AIB (ng g ⁻¹)	ISOVAL (ng g ⁻¹)	D/L-ISOVAL†	AIB/ISOVAL
+2.7 m	0.01	<0.2	<0.2	—	—
+1.2 m	0.01	<0.2	<0.2	—	—
+0.3 m	0.4	200	50	0.8, 0.94	4
K/T	87	<0.2	<0.2	—	—
-0.5 m	0.03	350	120	0.8, 0.96	3
-1.0 m	0.03	37	20	0.8, —	2
-2.2 m	0.03	<1	<1	—	—
-2.6 m	0.03	<0.2	<0.2	—	—
-4.4 m	—	<0.2	<0.2	—	—
-5.4 m	—	<0.2	<0.2	—	—

* Taken from ref. 4.

† The order of elution of the enantiomers is not known, so this could be the L/D ratio. Two enantiomer ratios are given. The first value was determined by the OPA/NAC HPLC method; co-elution of an unknown compound with one of the enantiomers probably accounts for the ratio which is not quite racemic. The second value was determined by the GC/MS specific ion monitoring technique and is considered to be the more accurate value, and to have an uncertainty of ± 0.02 .

FIG 2 GC/MS analysis with specific ion monitoring of the N-trifluoroacetyl-S-(+)-2-butyl ester derivatives of AIB and ISOVAL of same sediment extracts shown in Fig 1. *a* and *b* are a portion of the mass-154 ion chromatogram for authentic AIB and the AIB fraction from the sediment. Mass 154 is the diagnostic ion fragment (for example $(\text{CH}_3)_2\text{CNH}(\text{COCF}_3)$) for AIB. *c* and *d* are a portion of the mass-168 ion chromatogram for authentic racemic ISOVAL and the ISOVAL fraction from the sediment. Mass 168 is the diagnostic ion fragment (for example, $\text{CH}_3\text{CH}_2(\text{CH}_3)_2\text{CNH}(\text{COCF}_3)$) for ISOVAL. The D/L ISOVAL ratio of the standard compound was used to correct the ratio measured in the sediment fraction (see Table 1). A Hewlett-Packard 5988A GC/MS was used for the analyses. Separation of the derivatives was carried out on a fused-silica SE-54 column with splitless injection. The oven temperature was held at 40 °C for 1 min and then programmed at 10 °C min⁻¹ to 80 °C, 2 °C min⁻¹ to 120 °C, and finally 25 °C min⁻¹ to 250 °C. Helium was the carrier gas.



by volcanic processes is difficult to imagine because the abiotic synthesis of amino acids, or any other type of organic compound, has never been demonstrated under high-temperature, oxidizing conditions. At the high temperatures associated with volcanism, amino acids are rapidly destroyed rather than synthesized³³. The explanation that is most consistent with all of the evidence is that AIB and ISOVAL in the Stevns Klint sediments represent components of a bolide that collided with the Earth about 65 million years ago. The fact that the AIB/ISOVAL ratios (see Table 1) in the Stevns Klint sediments are within the same range as measured in the Murchison meteorite (AIB/ISOVAL = 2–4) supports this explanation. Thus, some fraction of the amino acids in the K/T impactor survived the impact event and are still preserved today.

One difficulty with our results is that the amount of AIB and ISOVAL in the K/T boundary layers is much larger than would be expected from a Murchison-like bolide, especially when one considers that some fraction of the amino acids would have been destroyed during impact. If we assume that all of the AIB in the Stevns Klint deposits was originally in the boundary clay, then we can estimate the AIB/Ir ratio at the time of deposition. The extraterrestrial amino acid signal appears to be distributed

over ~1 m above and below the K/T boundary; no signal is found in the boundary clay, but it is only a few centimetres thick. Using an average concentration of 100 ng g⁻¹ for the AIB-containing sediments, and a sediment density of 2.5 g cm⁻³, gives 5×10^{-5} g cm⁻² for the original AIB surface density, compared with a value of 4×10^{-7} g cm⁻² for Ir (ref. 1). We therefore estimate that the original AIB/Ir ratio in the K/T boundary layer was ~100, compared with a value of 20 for Murchison^{22,23}. The AIB/Ir ratio in the impactor would have been >100 because the amino acids would be partly decomposed on impact. Our results thus suggest that the K/T bolide was richer in extraterrestrial amino acids than Murchison, and/or it was depleted in Ir in comparison to most meteorites. Comets may fulfill this requirement. Alternatively, our results may imply that extensive abiotic synthesis of amino acids took place during the impact event, although this seems unlikely under oxidizing atmospheric conditions.

Further analyses are required to ascertain whether the extraterrestrial amino acid signal is present in other K/T boundary sequences, what processes may have subsequently disturbed these amino acids, and what kind of object supplied this unique organic cosmochemical impact signal.

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1. Alvarez L. W. et al. *Science* **208**, 1095–1108 (1980)
2. Sepkoski J. J. & Raup D. M. in *Dynamics of Extinction* (ed. Elliott D. K.) 3–36 (Wiley New York, 1986)
3. Luck J. M. & Turekian K. K. *Science* **222**, 613–615 (1983)
4. Kastner M. et al. *Science* **226**, 137–143 (1984)
5. Bohor B. F. et al. *Science* **224**, 867–869 (1984)
6. Wolbach W. S., Lewis R. S. & Anders E. *Science* **230**, 167–170 (1985)
7. Pollack J. B. et al. *Science* **219**, 287–289 (1983)
8. Alvarez W. et al. *Science* **223**, 1135–1141 (1984)
9. Bourgeois J. et al. *Science* **241**, 567–570 (1988)
10. Macdougall J. D. *Science* **239**, 485–487 (1988)
11. McHone J. F. et al. *Science* **243**, 1182–1184 (1989)
12. Officer C. B. & Drake C. L. *Science* **219**, 1383–1390 (1983)
13. Officer C. B. & Drake C. L. *Science* **227**, 1161–1167 (1985)
14. Hallam A. *Science* **238**, 1237–1242 (1988)
15. Brouwers E. M. et al. *Science* **237**, 1608–1610 (1987)
16. Padian K., Clemens W. A. in *Phanerozoic Diversity Patterns* (ed. Valentine J. W.) 41–96 (Princeton University Press, 1985)
17. Cronin J. R., Pizzarello S. & Cruikshank D. P. in *Meteorites and the Early Solar System* (eds Kerridge J. F. & Matthews M. S.) 819–857 (University of Arizona Press, Tucson, 1988)

18. Kvenvolden K. A., Lawless J. G. & Ponnamperuma C. *Proc. natn. Acad. Sci. U.S.A.* **68**, 486–490 (1971)
19. Chapman C. R. *Geochim. cosmochim. Acta* **40**, 701–719 (1976)
20. Kissel J. & Krueger F. R. *Nature* **326**, 755–760 (1987)
21. Hayatsu R. & Anders E. *Top. Curr. Chem.* **99**, 1–37 (1981)
22. Cronin J. R. & Pizzarello S. *Adv. Space Res.* **3**, 5–18 (1981)
23. Krahenbuhl U. et al. *Geochim. cosmochim. Acta* **37**, 1353–1370 (1973)
24. Das M. K., Raghobama S. & Balaram P. *Biochem. J.* **25**, 7110–7117 (1986)
25. Pollock G. E. et al. *Geochim. cosmochim. Acta* **39**, 1571–1573 (1975)
26. Roach M. C. & Harmony M. D. *Analyt. Chem.* **59**, 411–415 (1987)
27. Cheng Y. F. & Dovichi N. J. *Science* **242**, 562–564 (1988)
28. Gilmour I. & Anders E. *Geochim. cosmochim. Acta* **53**, 503–511 (1989)
29. Wall J. S. *Analyt. Chem.* **25**, 950–953 (1953)
30. Aswad D. *Analyt. Biochem.* **137**, 405–409 (1984)
31. Nimura N. & Kinoshita T. *J. Chromatogr.* **352**, 169–177 (1986)
32. Cronin J. R., Pizzarello S. & Gandy W. E. *Analyt. Biochem.* **93**, 174–179 (1979)
33. Miller S. L. & Bada J. L. *Nature* **334**, 609–611 (1988)

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Selective deficit of visual search in moving displays after extrastriate damage

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A VISUAL cue that is often associated with significant stimuli, such as those provided by prey and predators, is movement relative to the observer. An efficient visual system should be able to direct attention to those parts of the visual field that contain such stimuli. What is needed is a system that can filter by movement difference. This could direct attention to a moving item among stationary items, or an item moving in one direction against a background moving in a different direction. Visual search experiments have shown that people are indeed able to filter by movement; that is, they can attend to just the moving items in arrays of moving and stationary stimuli¹. Single-cell recordings from monkey visual cortex show that the medial temporal cortical area (MT) has some of the properties required to filter by movement²⁻⁴. We have now linked these two observations by showing that a patient with bilateral lesions to the presumed human homologue of MT cannot restrict visual attention to the moving items in arrays of both moving and stationary items. This suggests that MT is the site of a movement filter used in normal visual processing.

We have examined a patient, L.M., with a bilateral lesion to cortical visual-processing areas. By extrapolation from primate anatomy and on the basis of positron-emission tomography scans in humans (J. Allman, personal communication), this includes a region believed to be the human homologue of area MT in monkeys. She is a rare case, displaying a disturbance in tasks involving the detection and discrimination of visual movement, while retaining relatively unimpaired performance on many tasks involving form and colour. (Ref. 5 contains a detailed case study.) This selective deficit suggests damage to a sub-system specialized for the processing of some aspect of moving stimuli.

Our attempt to understand L.M.'s deficit, and thus the normal function of the damaged movement sub-system, used the logic introduced by Treisman and Gelade⁶. They required subjects to search for a target among varying numbers of non-targets. If detection time was independent of the number of stimuli present, they inferred that the feature(s) defining the target could be detected in parallel across the visual array. But if detection time increased linearly with the number of non-targets, they inferred that the target defining feature(s) could only be detected by a serial search through each item in the array.

It is well established that targets defined by a single salient feature can be detected in parallel. In contrast, targets defined by a conjunction of such features often require serial search⁶. (An example of single-feature search would be a search for an X among Os, or for a red object among green. An example of conjunction search is a search for a red X among red Os and green Xs, that is, the target is defined by a conjunction of form and colour.) It is known, however, that normal subjects can search in parallel for a moving X among intermingled moving Os and static Xs, even though this target is defined by a conjunction of form and movement¹. This result suggests the operation of a movement filter representing only the moving stimuli and excluding the static Xs. Among the moving stimuli the target is uniquely defined by its shape (an X among Os). Provided the movement filter is capable of making this form discrimination, or of specifying the coordinates of the moving objects to another

area that is capable, then search for the moving target will be parallel. If the movement filter is damaged in L.M., she will be unable to exclude static items when searching for a moving target. Her search time for targets defined by a conjunction of movement and form should increase with set-size because she will be distracted by the static non-targets which are the same shape as the moving target.

We first examined whether L.M. could search in parallel for targets defined by movement alone, and form alone. An inability to do this would obviously preclude detection of their conjunction in parallel, without providing insight into the operation of the movement filter. We asked L.M. to detect the presence of a single moving X in a display of stationary Xs (see Fig. 1a); her detection times are shown in Fig. 2. Although her performance is slower than normal, there is little effect of set-size and she can detect movement (at 1°s^{-1}) in parallel across the display. Figure 2 also shows her reaction time to detect a simple form, the presence of a static X among a field of static dashes (see Fig. 1b). Again, the data show little effect of set-size. The important conclusion from these initial experiments is that L.M.'s visual deficit still allows her to make judgements about the presence of slow movement, or of simple forms, in parallel across the visual array. It is therefore reasonable to ask whether she can detect a target defined by a conjunction of these two features in parallel, like normal subjects.

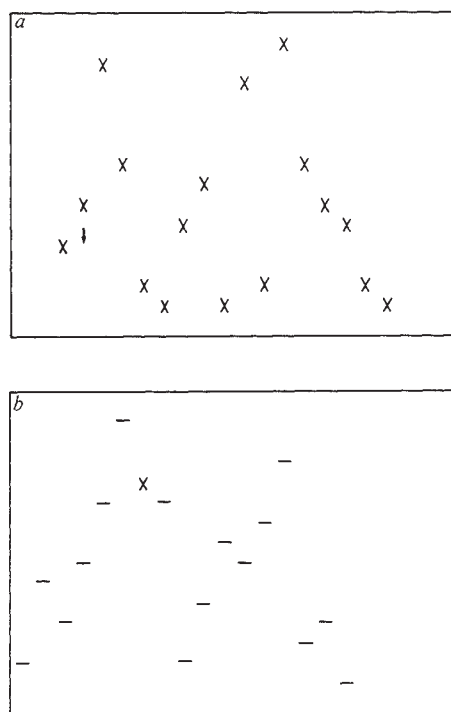


FIG. 1 Typical displays in the search for targets defined by a single feature (a, movement; b, form). The dotted arrow indicates a moving stimulus.

METHODS. L.M. faced a 36-cm enhanced graphics adaptor monitor, driven by a Tandon AT microcomputer, at a viewing distance of 1 m. The screen was divided into 25 imaginary columns. A different adjacent subset of these was used on each trial, with one stimulus in each. The vertical position of each stimulus within the column was chosen at random, but if a stimulus moved during the trial it started in the top half of the screen. The stimuli subtended $\sim 0.3^\circ$. Moving stimuli moved down the screen at 1°s^{-1} . On different blocks of trials the display contained 5, 9 or 17 items. L.M. held a single button. On blocks of trials providing the 'yes' data she had to respond on trials with a target and not on those without one. On blocks of trials providing the 'no' data she had to respond to the absence of a target and not on trials with a target. A target was present on 50% of 'yes' trials and 50% of 'no' trials. The procedure of running trials with go/no-go responses separately for 'yes' and 'no' data was adopted because L.M. had great difficulty in remembering which response went with which hand in the 2-choice design which is conventional with normal subjects.

In the conjunction experiment, L.M. was asked to detect a moving X among a field of randomly distributed moving dashes and stationary Xs (Fig. 3a). Her response times as a function of set-size are shown in Fig. 4. She shows a substantial effect of set-size, with a search time of roughly 160 ms per item (normals search such displays in parallel¹). Consistent with the hypothesis of a damaged movement filter, it seems that L.M. cannot restrict her search to the moving items. If she could, the target would effectively be defined by a form feature (X among dashes), which we know she can detect in parallel from the previous experiment.

In a control experiment the static Xs were replaced with static dashes (Fig. 3b). In this task, which was identical to the conjunction experiment in all other respects, the target (a moving X) was defined by its shape alone. Her detection time is shown in Fig. 4. Because there is little effect of set-size, it seems that she can search such displays in parallel. This demonstrates that her difficulty with the conjunction search in displays like that of

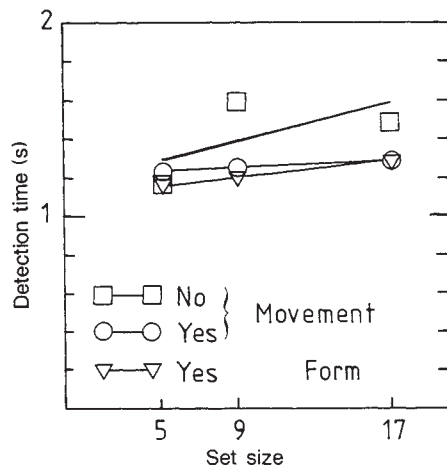


FIG. 2 Detection time as a function of set size for displays containing a single moving X among stationary Xs (Movement) or a single X among dashes (Form). Straight lines are least squares linear regressions.

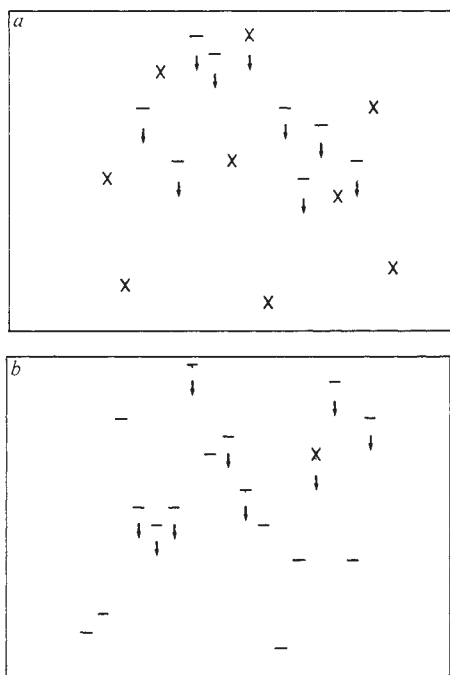


FIG. 3 Typical displays in the conjunction search experiment (a) and control experiment (b). L.M. was asked to detect the single moving X. In a, the target is defined by a conjunction of movement and form, whereas in b the target is defined by form alone in a display containing moving and stationary items.

FIG. 4 Detection of a single moving X among moving dashes and stationary Xs in displays like Fig. 3a (Os and □s), and in displays like Fig. 3b (▽s). In the conjunction search experiment (Fig. 3a), L.M. made 2% false positives on 'yes' trials (responding when there was no target), and 20% on 'no' trials. She missed 30% of targets (that is, she failed to respond within the 5-s viewing period) with a set size of 17, 8% with a set size of 9, and none with a set size of 5. This means that the real slopes of her set size versus detection-time functions must be greater than shown.

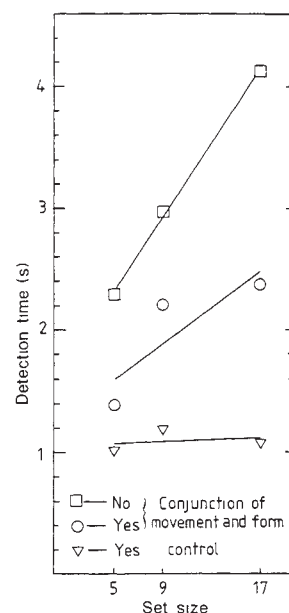


Fig. 3a is not simply a general problem with visual arrays where several stimuli move.

Search by normals in displays like that shown in Fig. 3a is unaffected by changes in the form of the static non-targets¹. This supports the claim that they detect the target by use of a movement filter, because static non-targets would not be represented there, and hence could not influence the search process. By contrast, L.M.'s performance was dramatically altered when the static Xs of the conjunction experiment were replaced by the static dashes of the control experiment. This confirms that one effect of her damage is an inability to exclude form information about static non-targets during search among moving items.

Two further controls eliminate alternative interpretations. First, because it might be argued that L.M.'s problem is with any conjunction search rather than with filtering by movement, we asked her to search for a target defined by a conjunction of colour and form—a green X among red Xs and green dashes. In an experiment similar to the one already described, but with the movement distinction replaced by one of colour, she showed a slope of 16 ms per item for 'yes' responses, and 39 ms per item for 'no' responses. This is within the normal range for such a task⁷, indicating that her problem is not with conjunction search *per se*, but specifically with conjunction search involving movement. Second, it is conceivable that L.M. can filter by movement but is not able to subsequently perform the necessary form discrimination on the moving stimuli. We therefore asked her to search for a stationary X among stationary dashes and moving Xs; that is, the original conjunction experiment was performed with the target as one of the stationary stimuli rather than one of the moving. Her search times were 88 ms per item for 'yes' response and 160 ms per item for 'no' responses, indicating that she finds the conjunction search for a stationary X as difficult as searching for the moving target. In conclusion therefore it seems that her problem is not an insensitivity to the presence and form of the moving items, but an inability to filter by motion.

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1. McLeod, P., Driver, J. & Crisp, J. *Nature* **332**, 154–155 (1988).
2. Felleman, D. & Kaas, J. *J. Neurophysiol.* **52**, 488 (1984).
3. Albright, T. *J. Neurophysiol.* **52**, 1106–1130 (1984).
4. Allman, J., Miezin, F. & McGuinness, E. *Perception* **14**, 105–126 (1985).
5. Zihl, J., von Cramon D. & Mai, N. *Brain* **106**, 313–340 (1983).
6. Treisman, A. & Gelade, G. *Cognit. Psychol.* **12**, 97–136 (1980).
7. Treisman, A. Q. *J. exp. Psychol.* **40**, 201–238 (1988).

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Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons

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INOSITOL 1,4,5-trisphosphate (InsP₃) mediates the effects of several neurotransmitters, hormones and growth factors by mobilizing Ca²⁺ from a vesicular, non-mitochondrial intracellular store¹. Many studies have indirectly suggested the endoplasmic reticulum (ER) to be the site of InsP₃ action²⁻⁶, though some have implicated the plasma membrane^{7,8} or a newly described smooth surfaced structure, termed the calciosome⁹. Using antibodies directed against a purified InsP₃-receptor glycoprotein¹⁰, of relative molecular mass 260,000, in electron microscope immunocytochemical studies of rat cerebellar Purkinje cells, we have now localized the InsP₃ receptor to ER, including portions of the rough endoplasmic reticulum, a population of smooth-membrane-bound organelles (smooth ER), a portion of subplasmalemmal cisternae and the nuclear membrane, but not to mitochondria or the cell membrane. These results suggest that in cerebellar Purkinje cells, InsP₃-induced intracellular calcium release is not the property of a single organelle, but is effected by specialized portions of both rough and smooth ER, and possibly by other smooth surfaced structures. The present findings are the first immunocytochemical demonstration of an InsP₃ receptor within a cell.

Polyclonal antiserum was prepared by injecting rabbits with InsP₃ receptor purified by column chromatography¹⁰. On western blots of cerebellar homogenate the antiserum recognized a single polypeptide of relative molecular mass (*M_r*) 260,000 (260K) (Fig. 1). At the light-microscope level, using the peroxidase technique, the InsP₃ receptor antibodies selectively labelled cerebellar Purkinje cells (Fig. 2a). Label was present throughout the perikaryon (with densest deposition in the immediate perinuclear region), and extended out to the finest branches of the tertiary dendrites. Other cerebellar neurons, such as granule cells, appeared unlabelled. Antiserum blocked with purified antigen yielded no specific label (Fig. 2b). The antibody also labelled neuronal perikarya in other areas of brain, including dorsal hippocampus, caudate nucleus and cerebral cortex (data not shown).

At the electron-microscope level, using the peroxidase pre-embedding technique, Purkinje cells were densely labelled (Fig. 3), whereas granule cells and non-neuronal elements were unlabelled. Portions of rough endoplasmic reticulum (RER) were heavily labelled (Fig. 3a-c), with those close to the nucleus near the dendritic pole labelled most consistently (Fig. 3b). Weakly labelled or unlabelled RER profiles were often immediately adjacent to heavily labelled profiles (Fig. 3c). In all Purkinje cells examined, the nuclear membrane was consistently labelled (Fig. 3a-c). In some sections, RER and extensions of nuclear membrane were in close proximity (Fig. 3a, b). Smooth

surfaced elements, including hypolemmal cisternae¹¹ near the cell membrane, were often labelled as well (Fig. 3d, e). In some cases, immunoreactive cisternae lay immediately subjacent to unlabelled presynaptic terminals (Fig. 3d); in other cases immunoreactive cisternae were near the cell membrane, but not subjacent to a presynaptic terminal (Fig. 3e). *Cis* cisternae of the Golgi apparatus were occasionally labelled, whereas intermediate and *trans* cisternae, as well as the *trans* Golgi network, were unlabelled (Fig. 3b). In dendrites, label was localized to ER-like structures (not shown). Mitochondria and cell membranes, however, consistently lacked specific label (Fig. 3a-e). Sections incubated with preimmune serum and processed at the same time showed no specific label (Fig. 3f) even when very close to the surface of the block.

As the heterogeneity of label might be considered an artefact of inconsistent penetration of antibodies into the section, Purkinje cells were also investigated by the ultrathin cryosection technique (data not shown). By this technique, RER cisternae were often labelled, but with varying density. Internal and external surfaces of membranes were labelled, as expected for a polyclonal antiserum that recognizes several determinants on the InsP₃ receptor glycoprotein, which presumably spans to ER membrane. Longitudinally sectioned stacks showed a density gradient of cisternae labelling which decreased away from the nucleus. Individual cisternae profiles were diffusely labelled, labelled at discrete sites or unlabelled. Labelling was also observed over a population of smooth surfaced, cytoplasmic

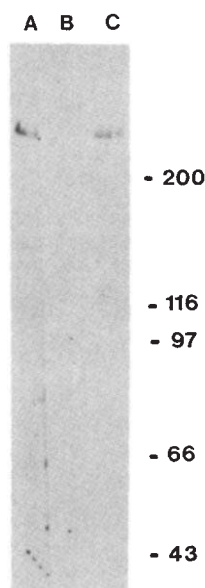


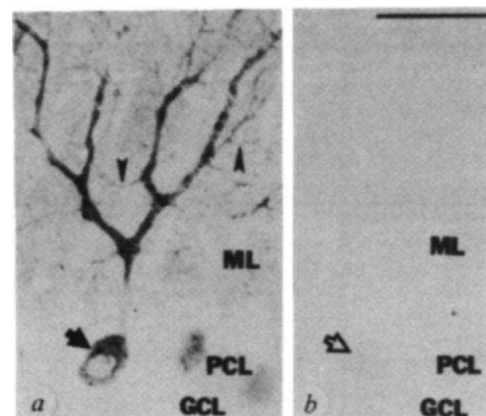
FIG. 1 Western blot analysis of InsP₃ receptor antibody recognition of receptor protein in cerebellar homogenate. Lane A, affinity-purified antibody incubated with blot of crude cerebellar homogenate transferred to nitrocellulose. Lane B, affinity-purified antibody pre-incubated with purified InsP₃ receptor protein incubated with blot of crude cerebellar homogenate. Lane C, affinity-purified antibody incubated with blot of purified InsP₃ receptor protein. Numbers refer to molecular weights of markers in thousands.

METHODS. InsP₃ receptor was purified by column chromatography¹⁰. Purified receptor (~100 µg) was emulsified with complete Freund's adjuvant and injected into each of three rabbits. Three and five weeks later, 100 µg receptor was injected with incomplete Freund's adjuvant, and after a further two weeks rabbits were bled. Antiserum was affinity-purified by incubation overnight with 50 µg purified antigen (immobilized in nitrocellulose after transfer from an SDS-polyacrylamide gel), eluted with 4 M MgCl₂ in 200 mM Tris-HCl buffer (pH 7.4), and dialysed against phosphate buffered saline with 0.1% Triton X100. For the western blot, 100 µg crude cerebellar homogenate protein (A and B) or 1 µg purified InsP₃ receptor protein (C) was separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Lanes were incubated with affinity-purified antibody (1:5) or affinity-purified antibody preblocked with 25 µg soluble purified InsP₃ receptor protein.

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FIG. 2 Light microscope immunocytochemical localization of InsP_3 receptor in rat cerebellar cortical Purkinje neurons. *a*, Labelled Purkinje cell after incubation of section with affinity purified antibody. Arrow, Purkinje cell body. Note the slightly denser accumulation of label surrounding the nucleus. Arrowheads, small labelled tertiary dendrites. Label in dendritic trees extended to the pial surface of the brain. GCL, granule cell layer; PCL, Purkinje cell layer; ML, molecular layer. *b*, Lack of label after incubation of sections with affinity-purified antibody blocked with purified antigen. Open arrowhead, unlabelled Purkinje cell body. (Scale bar, 100 μm .)

METHODS. Rats were perfused with 4% freshly depolymerized paraformaldehyde, and sagittal cerebellar sections were cut on a vibrating microtome. Sections were labelled by the avidin biotin technique²². Antiserum (rabbit no. 3, 1:400), preimmune serum (rabbit no. 3, 1:400), affinity-purified antibody (1:5), or blocked affinity-purified antibody (1:5) was applied overnight at 4 °C to free floating sections in 0.5 M Tris-0.9% NaCl (pH 7.4) and 1% normal goat serum. Blocked antiserum was prepared by incubating 1 ml diluted antiserum or affinity-purified antibody overnight at 4 °C with 10–25 μg purified antigen. Density of label was similar using antiserum or affinity purified antibody (data not shown). Labelled sections were examined with a Zeiss axioplan microscope with differential interference contrast optics.



organelles of various size and shape. Nuclear membrane, and occasionally *cis* portions of Golgi apparatus, were also labelled. Finally, label was present in smooth cisternae and profiles adjacent to the plasma membrane, probably representing portions of hypolemmal cisternae. Other organelles were unlabelled.

The immunocytochemical labelling was highly specific, as shown by the absence of label using preimmune serum or blocked antibody. In addition, two other antisera raised against the same antigen showed the same pattern of labelling at the light microscope level (data not shown). Label in the RER could represent polypeptide in the process of being synthesized; however, this is unlikely as label in the Golgi apparatus was restricted to occasional *cis* cisternae (the portion of Golgi most closely related to RER), rather than extending throughout the Golgi apparatus, as would be expected if the antibody were recognizing nascent peptide and polypeptide in transit through the Golgi¹². Furthermore, in various cells, InsP_3 releases calcium from heavy microsomes, presumably representing RER^{3,5,6}. Recent studies using subfractionation of microsomes demon-

strate that Ca^{2+} is released from the heavy fraction¹³. The regional distribution of InsP_3 binding and immunoreactive InsP_3 -receptor protein suggests that the antibody recognizes a physiologically relevant InsP_3 receptor. Label was most dense in Purkinje cells of the cerebellum, where both InsP_3 binding and InsP_3 -induced Ca^{2+} release are most enriched^{14,15}. Other areas of brain labelled by our antibody (dorsal hippocampus, caudate, certain areas of cerebral cortex) also show high levels of InsP_3 binding and InsP_3 -induced Ca^{2+} release^{14,15}. Although we cannot exclude the presence of this InsP_3 receptor in other neurons in the cerebellum, levels must be considerably lower than those in labelled cells.

Our recent reconstitution studies establish that the InsP_3 -receptor binding protein is physiologically associated with InsP_3 release of Ca^{2+} (C. Ferris *et al.*, in preparation). Vesicles comprising only lipids and purified InsP_3 -binding protein release $^{45}\text{Ca}^{2+}$, in a concentration-dependent fashion, in response to 0.1–10.0 μM Ins 1,4,5- P_3 , but not 10 μM Ins 1,3,4,5- P_4 or Ins 1,3,4- P_3 .

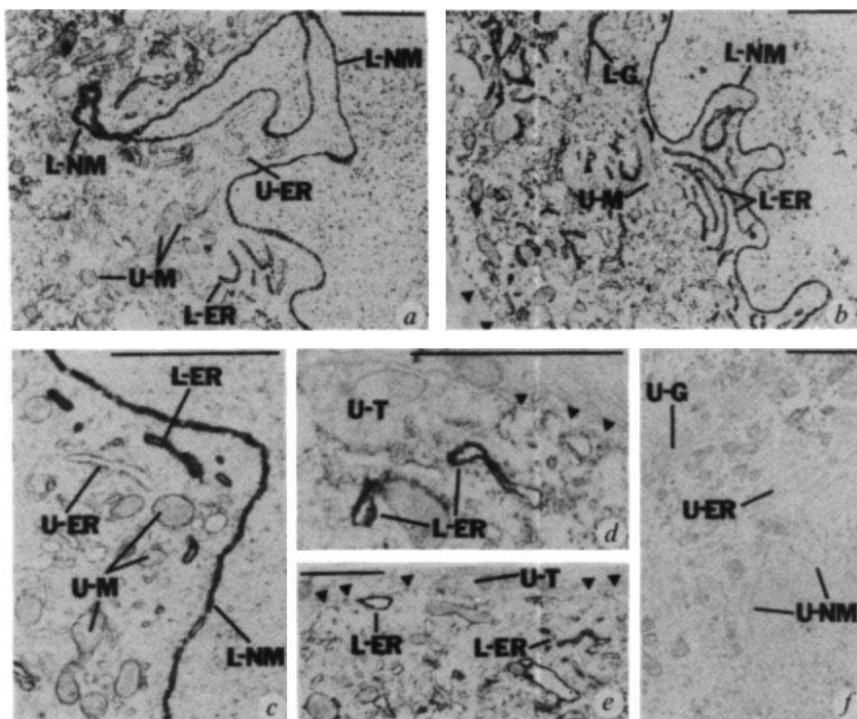


FIG. 3 Electron-microscope immunocytochemical localization of InsP_3 receptor in rat cerebellar cortex Purkinje neurons using pre-embedding avidin-biotin labelling^{22–24}. *a* and *b*, InsP_3 receptor antiserum. Nuclear membrane and some, but not all, portions of endoplasmic reticulum are labelled. L-ER, labelled endoplasmic reticulum; L-G, labelled Golgi apparatus; L-NM, labelled nuclear membrane; U-ER, unlabelled endoplasmic reticulum; U-M, unlabelled mitochondrion; U-NM, unlabelled nuclear membrane. *c*, InsP_3 receptor antiserum. Higher-magnification view of dendritic pole of labelled Purkinje neuron. Note the unlabelled ER very close to labelled ER (abbreviations as above). *d*, InsP_3 receptor antiserum. Labelled portions of endoplasmic reticulum (L-ER) immediately subjacent to an unlabelled presynaptic terminal (U-T). Triangles (*d*, *e*), cell membrane. *e*, InsP_3 receptor antiserum. Labelled portions of ER near plasma membrane, but not directly subjacent to presynaptic terminal. *f*, Preimmune serum. No specific label is present, even though the section is very close to the surface of the vibratome section. (Scale bars for all panels, 1 μm .)

METHODS. Vibrating microtome sections labelled with either InsP_3 receptor antiserum or pre-immune serum were prepared as described in Fig. 1. Sections were dehydrated and flat-embedded in Araldite between sheets of Aclar plastic. Regions of interest were selected and embedded in Beem capsules^{23,24}. Thin sections (500-Å thick) were cut, counterstained lightly in Reynolds' lead citrate and uranyl acetate, and examined in a Philips 201 electron microscope.

Previous suggestions for the localization of InsP_3 receptors in the ER were based on the enrichment of InsP_3 -induced Ca^{2+} release and InsP_3 binding in microsomes^{3,5,6}, which are composed primarily of ER elements. Density-gradient subfractionation of microsomes from different peripheral tissues or cultured cells has yielded variable results as to the subtype of ER involved. Some studies implicate heavy microsomes^{3,5,6,13,16}, which are enriched in RER elements; other studies implicate light microsomes¹⁷, which contain smooth ER elements as well as elements of the Golgi complex and other smooth-surfaced subcellular organelles. In a recent study with liver cells, InsP_3 binding and InsP_3 -induced Ca^{2+} release activity seemed to be higher in the plasma membrane⁷ than in the microsome fraction, but the plasma-membrane fraction may have been contaminated by associated cisternae. In a study using HL 60 cells, Ca^{2+} -release activity correlated with the distribution of a protein similar to calsequestrin (the Ca^{2+} -binding protein of the muscle sarcoplasmic reticulum), which has been localized within the calciosome, a newly described intracellular membrane-bound structure⁹. Although a calsequestrin-like protein is expressed by neurons (J.M. *et al.*, unpublished data), its ultrastructural localization has yet to be investigated.

InsP_3 receptors in distinct subcellular organelles may have diverse functions. Those in the subplasmalemmal cisternae, near presynaptic terminals, may mediate the postulated rapid Ca^{2+} mobilization induced by InsP_3 in postsynaptic cells¹. Those in the nuclear membrane and adjacent RER might respond to InsP_3 generated by neurotransmitters, hormones, and growth

factors, influencing protein synthesis^{18,19}, or helping to transfer their information from the cell membrane to the nucleus^{20,21}. □

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- Berridge, M. J. *A. Rev. Biochem.* **56**, 159–193 (1987).
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. *Nature* **306**, 67–69 (1983).
- Streb, H., Bayerdorffer, E., Haase, W., Irvine, R. F. & Schulz, I. *J. Membrane Biol.* **81**, 241–253 (1984).
- Joseph, S. K. & Williamson, J. R. *J. Biol. Chem.* **261**, 14658–14664 (1986).
- Muallem, S., Schoeffield, M., Pandol, S. & Sachs, G. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4433–4437 (1985).
- Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W. & Schulz, I. *J. Membrane Biol.* **81**, 69–82 (1984).
- Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. *J. Biol. Chem.* **263**, 4541–4548 (1988).
- Kuno, M. & Gardner, P. *Nature* **326**, 301–304 (1987).
- Volpe, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 1091–1095 (1988).
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. *J. Biol. Chem.* **263**, 1530–1534 (1988).
- Palay, S. L. & Chan-Palay, V. *Cerebellar Cortex, Cytology and Organization* (Springer, New York, 1974).
- Rothman, J. E. *Science* **213**, 1212–1219 (1981).
- Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. *Nature* (submitted).
- Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. *Nature* **325**, 159–161 (1987).
- Supattapone, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 8747–8750 (1988).
- Delfert, D. M., Hill, S., Pershadisingh, H. A., Sherman, W. R. & McDonald, J. M. *Biochem. J.* **236**, 37–44 (1986).
- O'Rourke, F. A., Halenda, S. P., Zavoico, G. & Feinstein, M. B. *J. Biol. Chem.* **260**, 956–962 (1985).
- Nairn, A. C., Nichols, R. A., Brady, M. J. & Palfrey, H. C. *J. Biol. Chem.* **262**, 14265–14272 (1987).
- Nairn, A. C. & Palfrey, H. C. *J. Biol. Chem.* **262**, 17299–17303 (1987).
- Goelet, P., Castellucci, V. F., Schachter, S. & Kandel, E. R. *Nature* **322**, 419–422 (1986).
- Berridge, M. *Nature* **323**, 294–295 (1986).
- Hsu, S.-M., Raine, L. & Fanger, M. *J. histochem. Cytochem.* **29**, 577–580 (1981).
- Miner, T. A., Aoki, C., Rex Sheu, K.-F., Blass, J. P. & Pickel, V. M. *J. Neurosci.* **7**, 3171–3190 (1987).
- Miner, T. A., Joh, T. H. & Pickel, V. M. *J. Neurosci.* **6**, 2585–2603 (1986).

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A combination of a particular HLA-DP β allele and an HLA-DQ heterodimer confers susceptibility to coeliac disease

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COELIAC disease is an autoimmune disease of the intestinal mucosa, elicited by ingestion of wheat gluten in genetically susceptible individuals¹. Susceptibility to coeliac disease has been associated with the serologically defined variants DR3 and DR7 of the class II antigens encoded by the HLA-D region^{2,3}. Three related class II antigens, each consisting of an alpha and a beta glycoprotein chain, have been identified and are designated HLA-DR, HLA-DQ, and HLA-DP. These highly polymorphic transmembrane proteins bind peptides derived from the processing of foreign antigens^{4–8} and present them to T lymphocytes; they also influence the specificity of the mature T-cell repertoire^{9–12}. The role of HLA-DP polymorphism in susceptibility has not been as fully explored as that of the other class II antigens because of the complexity of the primed lymphocyte typing (PLT) method for determining DPw specificities^{13–15}. Here we use a new DNA-based method of HLA-DP typing¹⁶ to analyse the distribution of $DP\beta$ alleles in a group of coeliac disease patients and healthy controls. Two specific $DP\beta$ alleles ($DPB4.2$ and $DPB3$) are increased in the patient population. Comparison of the $DP\beta$ sequences suggests

that the polymorphic residues at position 69 and at 56 and 57 may be critical in conferring susceptibility. Further, the contribution of the susceptible $DP\beta$ alleles appears to be independent of linkage to the previously reported DR3 and DR7 markers for coeliac disease. The distribution of $DQ\alpha$ and β alleles in patients suggests that a specific DQ heterodimer may be responsible for the observed DR associations. Individuals with both this DQ antigen and a specific $DP\beta$ allele are at increased risk for coeliac disease.

Molecular analysis has revealed the heterogeneity of the DR and DQ serotypes^{17,18} and, recently, restriction fragment length polymorphism (RFLP) markers associated with coeliac disease have been observed using $DP\beta$ ¹⁹, $DP\alpha$ ^{19,20} and $DQ\alpha$ ²¹ complementary DNA probes. Recently, we have developed a simple and highly informative method of typing DP polymorphism based on the polymerase chain reaction (PCR) amplification^{22–24} of the variable second exon of the $DP\beta$ locus¹⁶. In a study of 34 cell lines DPw-typed by PLT, we identified 14 alleles at the $DP\beta$ locus and 2 at the $DP\alpha$ locus¹⁶. Allelic sequence variation can be detected using a panel of oligonucleotide probes and each allele identified by a diagnostic pattern of probe hybridization. In this report, we have applied this method to examine the distribution of $DP\beta$ alleles in coeliac patients and in control individuals.

Sequence determination of PCR-amplified DNA, revealed that 3 of 4 coeliac disease patients contained the relatively rare allele, $DPB4.2$ (see Fig. 1). Subsequent analysis of additional patients and of healthy controls has identified an additional 7 $DP\beta$ alleles, bringing the total to 21 (Fig. 1a and b), in contrast to the 6 DPw specificities defined by PLT. $DPB13$ and $DPB14$ were observed in coeliac disease patients, whereas the alleles $DPB15$ – $DPB19$ were identified in control individuals and in cell lines. In general, these sequences represent new combinations of the previously described $DP\beta$ alleles. The second exon of the $DP\beta$ locus contains 6 variable regions with a limited number of polymorphic residues ($n = 2$ or 3) at each position. This patchwork pattern of polymorphism suggests that recombination may have played an important role in generating $DP\beta$ allelic diversity. These alleles can all be identified using a panel of 14 sequence-specific oligonucleotide (SSO) probes (see Fig. 1a legend). The distribution of $DP\beta$ alleles was determined by

TABLE 1 DR and DP β typings on coeliac disease patients

	Patient	DR	DQw	DP β
CD1	CaMa	3, 4	2, 3	10, 4.1
CD2	CiMa	7, 5	2, 3	4.1, 4.2
CD3	CoAn	7, —	1, 2	9, 13
CD4	IaDa	NT	NT	10, 4.2
CD5	InNu	3, 7	2, —	4.1, 4.2
CD6	IzMa	3, 5	2, 3	4.2, 3
CD7	RiTi	2, 3	1, 2	3, 14
CD8	BaAn	3, 7	2, 3	4.1, 4.2
CD9	CaBa	5, 7	2, 3	4.1, 4.2
CD10	CaFa	1, 4	1, 3	4.1, 8
CD11	CoVi	2, 3	1, 2	1, 4.2
CD12	CrCa	3, 5	2, 3	3, 2.1
CD13	GuMa	3, 4	2, 3	4.1, 4.2
CD14	IaGi	7, 8	2, 3	3, 4.1
CD15	IaFi	1, 4	1, 3	3, 4.1
CD16	IIAd	3, 5	2, 3	4.1, 4.2
CD17	GiGi	1, 3	1, 2	2.1, 3
CD18	MiAn	5, 7	2, 3	4.2, 2.1
CD19	PeLu	3, 7	2, 2	4.1, 3
CD20	SaEl	5, 7	2, 3	4.1, 4.2
CD21	NaSt	3, 6	1, 2	4.1, 4.1
CD22	RoLu	5, 7	2, 3	9, 14
Pat	EsLe	2, [5]	1, [3]	3, [4.2]
Mat	Es	5, [7]	[2], 3	[3], 5
CD23	EsDa	[5], [7]	[2], [3]	[3], [4.2]

NT indicates sample not types. Coeliac disease patients were diagnosed by clinical evidence of malabsorption, morphological criteria on small bowel biopsies, and by histological and clinical improvement on a gluten-free diet⁴. Serological HLA typing was performed by the standard microcytotoxicity assay. Genomic DNA was extracted from peripheral blood lymphocytes or from Epstein-Barr virus-transformed cell lines and amplified by PCR²²⁻²⁴ using *Taq* polymerase (Cetus); a Perkin-Elmer Cetus Thermal Cycler, the DP β PCR primers DB01 and DB03, as described¹⁶. The sequences of oligonucleotides used as hybridization probes for DP-typing are described in Fig. 1a. These probes were labelled with horseradish peroxidase and detected as previously described^{36,37} using the chromogen 3,3',5,5'-tetramethyl benzidine (Fluka). In the family of CD23, the DR, DQ, and DP alleles in boxes represent the paternal haplotype and the alleles in circles represent the maternal haplotype.

SSO probe analysis in 23 coeliac disease patients from Italy and in 19 ethnically matched controls (Tables 1 and 2), as well as in 150 other control individuals (Table 2).

The frequency of the *DPB4.2* allele is significantly increased in the coeliac disease patients relative to the ethnically restricted Italian controls (52% versus 10.5%; $P=0.005$) as well as the other controls. The calculated relative risk (RR=9.3) for the *DPB4.2* allele is higher than for the classic *DR3* and *DR7* markers (RR=2.8 and 3.0, respectively) for this group of patients. Similar risk estimates for *DR3* and *DR7* were reported by Tosi *et al.*²⁵. Six of the 11 (55%) non-*DPB4.2* patients carry the *DPB3* allele. Most (78%) of the coeliac disease patients carry either *DPB4.2* or *DPB3* compared with only 21% of the

Italian controls (Table 2). The relative risk for the presence of either of these markers is 13.5. The observation of a specific *DPB* allele associated with the disease could simply reflect linkage disequilibrium, with the *DPB4.2* (or *DPB3*) allele being a marker for a particular disease haplotype. Alternatively, the association could reflect the contribution of the *DPB* allele to susceptibility independent of the well-known *DR3* and *DR7* coeliac disease-associated haplotypes. The finding that the same proportion of *DR3*⁺ patients and *DR7*⁺ patients carry the *DPB4.2* and *DPB3* alleles as do all coeliac disease patients (Table 3) suggests that the association of these *DPB* alleles is independent of linkage to the *DR3* and *DR7* associated susceptibility haplotypes. In control haplotypes, there is no evidence for linkage disequilibrium between *DPB4.2* (or *DPB3*) and *DR3* or *DR7* (Table 2). In one pedigree tested, the *DPB4.2* allele in the patient was on a paternal *DR5* haplotype and the *DPB3* allele on a maternal *DR7* haplotype (Table 1). Another independent relationship between the disease-associated class II alleles *DPB2.1* and *DR5* and *DRw8* was observed in our recent study of pauciarticular juvenile rheumatoid arthritis²⁶.

Because most *DPw4* typed cells have the *DPB4.1* allele, not *DPB4.2*, and because many cells with the *DPB4.2* allele have been PLT typed as DP-blank¹⁶, it is not surprising that no DPw specificity has been previously associated with coeliac disease. In addition to its capability of subdividing immunologically or RFLP defined variants, PCR/SSO typing has the virtue of revealing how alleles differ. The *DPB4.2* allele differs from the *DPB2.1* allele only by a Lys→Glu change at position 69 (ref. 16, Fig. 1), suggesting that this residue may play a critical role in susceptibility to the disease. As discussed previously¹⁶, the charge of this residue must be important in T-cell recognition because cells with the *DPB4.2* allele (Lys 69) have the PLT-defined specificity DPw4 (or blank) whereas cells with *DPB2.1* (Glu 69) type as DPw2. The polymorphic residues in this region of the *DRB1* chain have also been implicated in susceptibility to insulin-dependent diabetes mellitus (IDDM) and pemphigus vulgaris (PV)²⁷⁻²⁹. Not all *DPB* alleles with Lys 69 confer susceptibility however (Fig. 1). It is the allelic structure of the beta chain, not simply an isolated residue that appears most highly associated with disease. The *DPB3* allele which is also associated with coeliac disease is present on over half (6/11) of the non-*DPB4.2* patients. This allele shares not only the Lys 69 with *DPB4.2* but is the only other common *DPB* allele with both Asp-Glu at positions 56-57 and Lys 69. (The newly discovered *DPB14* (allele frequency <0.02) and *DPB18* ($f<0.005$) alleles also encode these residues at positions 56, 57 and 69, but they are relatively rare.) The nature of the polymorphic residue at position 57 of the DQB chain appears to be an important element in susceptibility to IDDM and PV²⁷⁻³¹. In the structural model for class II antigens proposed by J. H. Brown *et al.*,³² the polymorphic residues at these locations point in towards the putative peptide binding cleft and may, therefore, serve as peptide contact sites.

Another study of DP polymorphism in a group of coeliac disease patients from the United States revealed a somewhat

TABLE 2 Frequency of specific DP β alleles in coeliac disease patients and controls

DP β alleles	Coeliac disease patients (n=23)	Italian controls (n=19)	General controls (n=150)	Relative risk
4.2	12 (52%)	2 (10.5%) $P=0.005$	22 (15%) $P<0.001$	9.3*, 6.3†
3	8 (35%)	3 (15.8%) $P=0.15$	18 (12%) $P=0.009$	2.8*, 3.9†
4.2 or 3	18 (78%)	4 (21%) $P<0.001$	40 (27%) $P<0.001$	13.5*, 9.9†

Statistical significance (P) was calculated by using the Fisher exact test (one-tailed) or the χ -square test. The distribution of DP β alleles in 150 control individuals, not selected for matching ethnicity, was not significantly different from the distribution in the ethnically restricted Italian control sample. These control samples included 50 blood samples from random unaffected individuals and 100 cell lines. Based on the analysis of all the DP β alleles by the heterogeneity test, the overall distribution of alleles in the patients and both control groups was different ($P<0.05$). The *DPB4.2* or *DPB3* alleles were the only markers whose frequencies were different between patients and both control groups. In the calculation of relative risk, * refers to the values calculated using the Italian control frequencies and † to the calculation using the general control frequencies.

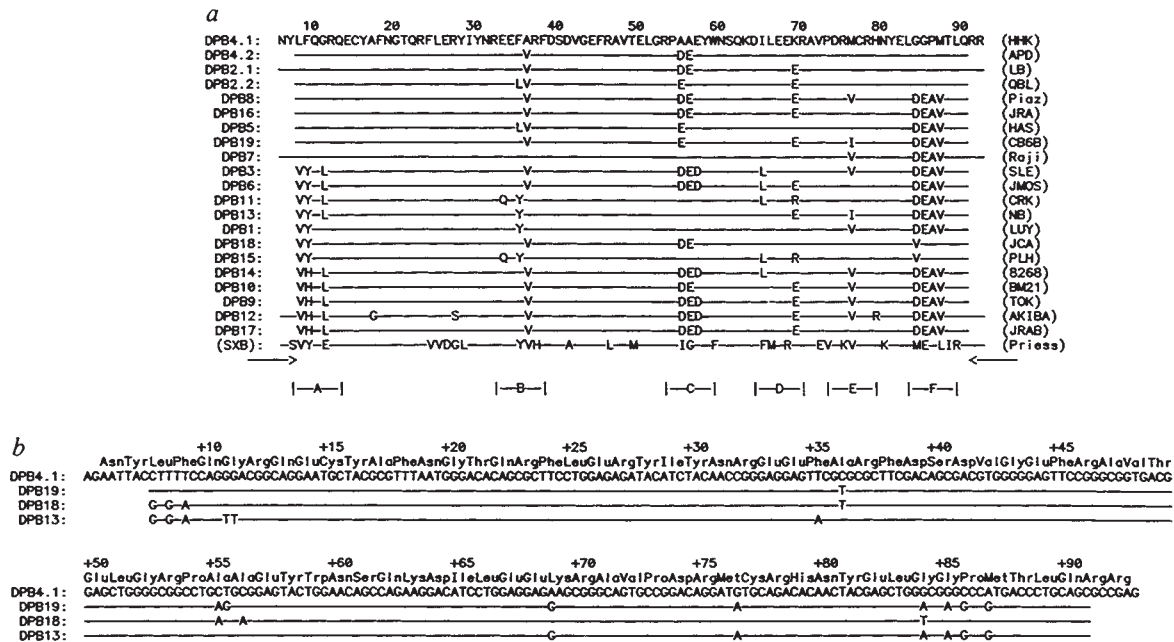


FIG. 1. *a*, HLA-DPB protein sequences. The nucleotide sequences were translated into the standard one-letter amino-acid code and aligned to the *DPB4.1* allele. The arrows indicate the positions of the PCR amplification primers and the letters show the six variable regions. The designations of the allele are shown at left and the representative sources indicated at the right. The sequences of *DPB1–DPB12* have been previously reported¹⁶. Probes for region A are the following: DB10 (GAATTACCTTTCCAGGGA) specific for L-F-Q-G, DB11 (ATTACGTGTACCACTTACG) for V-Y-Q-L, DB12 (CGTCCCTGGTACAGTAAT) for V-Y-Q-G and DB22 (ATTACGTGCAC-CAGTTACG) for V-H-Q-L. Probes for region C are DB13 (CCTGCTCGGAG-TACTG) specific for A-A-E, DB14 (CAGTACTCTCATCAGG) for D-E-E, DB16 (CAGTACTCCGCTCAGG) for E-A-E, and DB17 (CCTGATGAGGACTACTG) for D-E-D. Probes for region D are DB18 (GACATCTGGAGGAGAAGC) specific for L-L-E-E-K, DB19 (GCTCTCTCCAGGATGTC) for L-L-E-E-E, DB20 (GACCTCTGGAGGAGAAGC) for L-L-E-E-K, and DB21 (GCTCTCTCCAG-

GAGGTC) for L-L-E-E-E. Probes for region F are DB25 (CTGCAGGGTCATGGCCCCCG) specific for G-G-P-M and DB26 (CTGCAGGGTCACGGCCTCGTC) for D-E-A-V. These sequence-specific probes are labelled with horseradish peroxidase at the 5' end. *b*, Nucleotide sequences of *DPB13*, *DPB18* and *DPB19* aligned with *DPB4.1*. The sequences of *DPB14–DPB17* will be reported elsewhere²⁶. One microgram of DNA was PCR amplified for 30 cycles by *Taq* polymerase with primers DB01 and DB03 (ref. 16). The PCR reaction was extracted twice with equal volume of phenol:chloroform, and spin-dialysed for one hour at 5,500 r.p.m. through Centricon 10 in 2 ml of water. The DNA was digested with *Bam*HI and *Pst*I, phenol:chloroform extracted, and spin-dialysed again through Centricon 10. 10% of the digested DNA was ligated into the m13mp10 vector and clones were screened with a [³²P]-labelled *DPB* cDNA probe. Positive clones were purified and sequenced by the dideoxy chain termination method.

different pattern of *DPB* association³³. In these patients, who were predominantly *B8*, *DR3* with no significant increase in *DR7*, an increase in the *DPB1* allele frequency as well as that of the *DPB3* was observed. A slight increase in the *DPB4.2* allele was also observed; half of the non-*DPB1* or *DPB3* patients were *DPB4.2*. Thus, in different ethnic groups, different *DPB* alleles may be associated with susceptibility to coeliac disease. In both studies, however, virtually all the patients have the *DQw2* serotype. Whereas 78% (18/23) of the patients in this study are *DPB4.2* or *DPB3*, 91% (20/22) are *DQw2* (*DR3* or *DR7*). *DR3* and *DR7* haplotypes each have the *DQB2* allele at the *DQB* locus²⁹ which encodes the *DQw2* specificity. This

observation suggests that the *DQ* antigen is a major determinant of susceptibility and that specific combinations of *DQB* alleles (*DQB2*) and *DPB* alleles (*DPB4.2* or 3) may confer increased risk for coeliac disease. A specific *DQα* allele (*DQA4*) common to *DR3*, *DR5*, and *DRw8* haplotypes²⁹ has also been implicated in disease susceptibility by serological²⁵, RFLP²¹, and more recently, by oligonucleotide³⁴ studies. Thus, the same *DQ* heterodimer (*DQB2* and *DQA4*) is encoded in *cis* by the *DR3* haplotype and in *trans* by the *DR5/DR7* or the *DRw8/DR7* genotypes. (The sequence of the *DQα* allele (*DQA4.1*) on *DR3* and *DR5* haplotypes is identical and differs from the sequence of the *DQα* allele on *DRw8* haplotypes (*DQA4.2* or *DQA4.3*) by only two or three amino acids²⁹.) Of the 11 *DR7*⁺ patients (Table 1), 6 were *DR5/DR7* heterozygotes compared with an expected number of 2 based on the *DR5* allele frequency in patients (*P* < 0.05). Of the other 5 *DR7*⁺ patients, 3 had *DR3*, and one had *DRw8* on the other haplotype. Of the 9 *DR5*⁺ patients (Table 1), 6 had *DR7* and 3 had *DR3* on the other haplotype. This distribution is consistent with the notion that it is this particular *DQ* heterodimer (*DQB2* and *DQA4*), in combination with the *DPB4.2* (or *DPB3*) allele, that confers high risk for coeliac disease. Similarly, it seems that specific combinations of *DQB* alleles (*DQB3.2*) and *DRB1* alleles (*Dw4* or *Dw10*) account for most of the observed *DR4* association with IDDM^{28,35}. This putative joint contribution of different class II molecules could be mediated at the level of peptide binding and presentation, at the level of T-cell repertoire, or at both. Whatever the mechanism, polymorphic residues of the *DPB* chain, as well as those of the better characterized *DR* and *DQ* antigens, seem to confer susceptibility to autoimmune diseases. □

TABLE 3 Independence of specific <i>DPB</i> alleles and DR serotypes			
Frequency of <i>DPB</i> alleles in DR-typed patients			
	DR3 ⁺	DR7 ⁺	All coeliac disease
Frequency of <i>DPB4.2</i> allele	6/12 (50%)	7/11 (64%)	12/22 (54%)
Frequency of <i>DPB3</i> allele	6/12 (50%)	3/11 (27%)	8/22 (36%)
Frequency of <i>DPB</i> alleles on control DR3 and DR7 haplotypes			
	Control DR3	Control DR7	All control
Frequency of <i>DPB4.2</i> allele	2/21 (10%)	1/14 (7%)	12/97 (12%)
Frequency of <i>DPB3</i> allele	4/19 (21%)	1/13 (8%)	10/97 (10%)

The determination of DP/DR haplotypes was carried out by analysing 97 haplotypes from homozygous typing cell lines.

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1. Booth, C. C. in *Proceedings of the 2nd International Coeliac Symposium* (ed. Stenfort Kroese) 17 (1974).
2. Tiwari, J. L. & Terasaki, P. I. (eds) *HLA and Disease Associations* (Springer, New York, 1985).
3. De Marchi, et al. *Tissue Antigens* **14**, 309–316 (1979).
4. Babbitt, B. P., Allen, P. M., Matsueda, G., Huber, E. & Unanue, E. *Nature* **317**, 359–361 (1985).
5. Watts, T. M. & McConnell, H. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9660–9664 (1986).
6. Unanue, E. R. & Allen, P. M. *Science* **236**, 551–557 (1987).
7. Guillet, J.-G. et al. *Science* **235**, 865–870 (1987).
8. Sette, A. et al. *Nature* **328**, 395–399 (1987).
9. Kappler, J. W., Roehm, N. & Marrack, P. *Cell* **49**, 273–280 (1987).
10. Kappler, J. W., Staerz, U., White, J. & Marrack, P. C. *Nature* **332**, 35–40 (1988).
11. MacDonald, H. R. et al. *Nature* **332**, 40–46 (1988).
12. Teh, H. S. et al. *Nature* **335**, 229–233 (1988).
13. Mawas, C., Charnot, D. & Mercier, P. *Tissue Antigens* **15**, 458–466 (1980).
14. Wink, R., Schendel, D., Hansen, J. A. & Dupont, B. *Immunogenetics* **4**, 107–115 (1978).
15. Shaw, S., Johnson, A. H. & Shearer, G. M. *J. exp. Med.* **152**, 565–580 (1980).
16. Bugawan, T. L. et al. *J. Immunol.* **141**, 4024–4030 (1988).
17. Trowsdale, J. et al. *Immunol. Rev.* **85**, 5–43 (1985).
18. Erlich, H. A., Horn, G. T., Bugawan, T. L. & Scharf, S. J. in *Molecular and Cellular Biology of Histocompatibility Antigens* (eds Schacter, B., et al.) 93–109 (ASHI, New York, 1987).
19. Howell, M. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 222–226 (1988).
20. Niven, M. J. et al. *Lancet* **i** 805 (1987).
21. Roep, B. O. et al. *Hum. Immun.* **23**, 271–279 (1988).
22. Saiki, R. K. et al. *Science* **230**, 1350–1354 (1985).
23. Mullis, K. & Faloona, F. *Meth. Enzym.* **155**, 335–350 (1987).
24. Saiki, R. et al. *Science* **239**, 487–491 (1988).
25. Tosi, et al. *Clin. Immun. Immunopath.* **28**, 395–404 (1983).
26. Begovich, A. et al. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
27. Scharf, S. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3504–3508 (1988).
28. Erlich, H. A., Horn, G., Scharf, S. J. & Bugawan, T. in *Molecular Biology of HLA Class II Antigens* (ed. Silver, J.) (CRC in the press).
29. Horn, G., Bugawan, T., Long, C. & Erlich, H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6012–6016 (1988).
30. Todd, J. A., Bell, J. I. & McDevitt, H. O. *Nature* **329**, 599–604 (1987).
31. Sinha, A. A. et al. *Science* **239**, 1026–1029 (1988).
32. Brown, J. H. et al. *Nature* **332**, 845–850 (1988).
33. Kagnoff, M. et al. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
34. Sollid, L. M. et al. *J. exp. Med.* **169**, 345–350 (1989).
35. Sheehy, et al. *J. clin. Invest.* **83**, 830–835 (1989).
36. Saiki, R. et al. *New Engl. J. Med.* **319**, 537–541 (1988).
37. Bugawan, T. L., Saiki, R. K., Levenson, C. H., Watson, R. M. & Erlich, H. A. *BioTechnology* **6**, 943–947 (1988).

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Physical association between MHC class I molecules and immunogenic peptides

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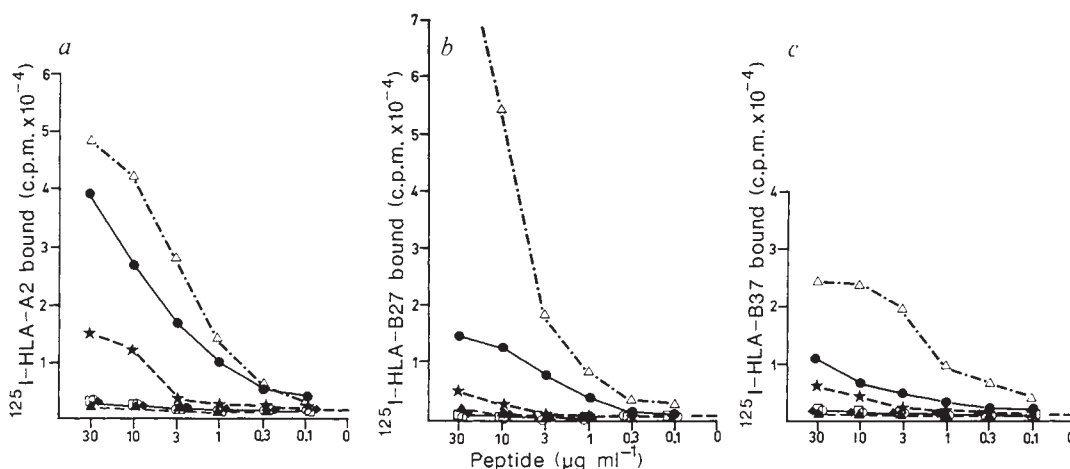
ANTIGENIC peptides are presented to T lymphocytes by major histocompatibility complex (MHC) molecules^{1–14}. The binding of peptides to MHC class II molecules has been demonstrated directly, and is found to correlate with the ability of specific class II alleles to restrict the T-cell response to specific peptides^{2–8}. By comparison, a direct demonstration of a physical association between antigenic peptides and MHC class I molecules has proved difficult. A recent report¹⁵ shows that it is possible, however, and the three-dimensional structure of a class I MHC molecule^{16,17}

illustrates the site where such binding must occur. Here we describe a simple assay which measures the binding of radiolabelled MHC class I molecules to peptides bound to a solid phase support. We find that class I molecules bind specifically to peptides known to be antigenic for class I-restricted cytotoxic T lymphocytes. Peptides which are recognized by cytotoxic T lymphocytes bind not only to the restricting MHC class I molecule but also to other class I molecules. Our results suggest that quantitative differences in the peptide/MHC class I interaction may influence the pattern of MHC restriction observed *in vivo*.

In preliminary experiments we tried to demonstrate peptide binding to MHC class I molecules by methods which have been fruitful for the class II system, including equilibrium dialysis and isolation of labelled peptide/MHC molecules complexes by gel filtration^{1–8}. We were not able to obtain reproducible results with this approach. By contrast, reproducible results were obtained using a solid phase assay in which the wells of microtitre plates are coated with synthetic peptides and the binding of iodinated purified human MHC class I (HLA) molecules measured. Three reference peptides, known to be targets for antiviral cytotoxic T lymphocytes (CTL), were selected: (1) M.55–71, a peptide from the influenza A matrix protein which is recognized by HLA-A2 restricted CTL (ref. 10) (and four derived peptides, see Table 1); (2) N.335–349Y⁺, a peptide from the nucleoprotein of the same virus, which is recognized by HLA-B37 restricted CTL (ref. 9); and (3) the HIV-1 gag peptide

FIG. 1 Binding of HLA class I molecules to various concentrations of immobilized peptides. Total amount of [¹²⁵I]-HLA-A2(a), [¹²⁵I]-HLA-B27 (b) and [¹²⁵I]-HLA-B37 (c) molecules bound to wells coated with the peptides M.Y*57–68 (●), N.335–349Y⁺ (★), G.265–279C⁺ (△), A2.139–159 (□), G.205–219C⁺ (▲) and N.147–158R⁺ (◆) and F.161–175 (○).

METHODS. Wells of microtitre plates were either coated with 100 µl of peptide (M.Y*57–68, F.37–50, A2.139–159) diluted in phosphate buffered saline (PBS), pH 7.4, for 16 h at 4 °C or pretreated with 2.5% glutaraldehyde diluted in water for 2 h at room temperature and then coated with 100 µl of peptide in carbonate-bicarbonate buffer, pH 9.6, for 16 h at 4 °C. Remaining free sites were saturated by incubation with 1% bovine serum albumin (BSA) for 2 h at 20 °C. After extensive washing, purified [¹²⁵I]-HLA molecules (100 µl containing 1–3 × 10⁵ c.p.m., 10^{–9} M)



diluted in PBS containing 0.05% Tween 20, 1% BSA, 0.02% sodium azoture, 1 mM PMSF and 10 µg ml^{–1} trypsin inhibitor (Sigma) were added and the plates were incubated for 20 h at 20 °C. After further extensive washing, the radioactivity in each well was counted. Class I molecules had been purified by affinity chromatography as described by Walker¹⁹ with some modifications²⁰.

TABLE 1 Peptides used for binding to MHC class I molecules

Synthetic peptides	Amino-acid sequence	CTL restricting element	-A2	Direct binding to HLA -B27	-B37
Influenza A virus					
Matrix					
M.55-71	LTGILGFVFTLTPSE	HLA-A2	++	+	+
M.56-68	TKGILGFVFTLTV	HLA-A2	++	+	+
M.Y ⁺ 57-68	YKGILGFVFTLTV	HLA-A2	++++	+	+
M.57-68	KGILGFVFTLTV	HLA-A2	++	+	+
M.62-68	FVFTLTV	—	—	—	—
Nucleoprotein					
N.335-349Y ⁺	SAAFEDLRVLSFIRGY	HLA-B37	++	+	+
N.147-158(R156 ⁻)	TYQRTRALVTG	H-2K ^d	—	—	—
HIV-1					
Gag					
G.265-279C ⁺	KRWIILGLNKIVRMYC	HLA-B27	+++++	+++++	+++
G.255-269C ⁺	NPPIPVGEIYKRWIIC	—	—	—	—
G.205-219C ⁺	ETINEEAAEWDRVHPC	—	—	—	—
Nef					
F.37-50	LEKHGAISSNTAA	—	—	—	—
F.161-175	ENTSLHPVSLHGMD	—	—	—	—
Vpr					
R.68-80	LFIHFRIGCRHSR	—	++++	++++	+++++
Class I molecules					
HLA-A2					
A2.98-113	MYGCDVGSDWRFLRGY	—	—	—	—
A2.139-159	AAQTTHKKWEAAHVAEQLRAY	—	—	—	—
A2.170-185	RYLENGKETLQRTDAP	H-2K ^d	—	—	—

G.265-279C⁺, which is recognized by HLA-B27 restricted CTL (ref. 18). Figure 1a shows that M.Y⁺57-68 derived from M.55-71 bound especially well to HLA-A2. It also bound to HLA-B27 and HLA-B37, although at a much weaker level (Fig. 1b, c). G.265-279C⁺ strongly reacted with HLA-A2 and -B37 and still more efficiently with HLA-B27 (Fig. 1a, b, c). N.335-349Y⁺ reacted weakly with all HLA molecules (Fig. 1a, b, c). Several other peptides, not known to be CTL epitopes, gave completely negative results (Table 1). Thus the three reference peptides bound to HLA class I molecules whereas control peptides did not. Moreover, a preferential association was observed between M.Y⁺57-68 and HLA-A2 and between G.265-279C⁺ and HLA-B27, although it is clear that the antigenic peptides also reacted with other HLA molecules. Kinetic experiments showed that at 20 °C, a plateau was reached between 9 and 20 h (not shown), indicating that the association is faster than peptide binding to class II molecules^{1,2}. Competition experiments showed that ovalbumin, human immunoglobulins or β_2 -microglobulin were unable to compete for the fixation of HLA-A2 on M.Y⁺57-68, whereas different HLA class I molecules clearly did compete (Fig. 2).

To obtain more quantitative data, and to avoid possible artefactual binding of HLA molecules to the plates and/or variability arising from differential peptide binding to the plates, competition experiments with soluble peptides were performed. Iodinated HLA-A2 molecules were preincubated with free competitor peptides derived from M.55-71 and inhibition of binding to M.Y⁺57-68 coated wells was then tested. Peptide M.Y⁺57-68 itself competed more efficiently than M.55-71, M.56-68 or M.57-68, and M.62-68 was much less efficient (Fig. 3a). When the same peptides were tested in a cytolytic assay, a correlation was found between the binding of HLA-A2 to a given peptide and its capacity to sensitize HLA-A2⁺ cells for lysis by anti-influenza A virus CTL (Fig. 3c). When unrelated peptides were compared in the binding competition assay, not only M.Y⁺57-68 but also N.335-349Y⁺ and G.265-279C⁺ inhibited the binding of HLA-A2 to solid phase M.Y⁺57-68 and N.335-349Y⁺ (Fig. 3a, b) and also the binding of HLA-B27 to G.265-279C⁺ (not shown). G.265-279C⁺ was the most efficient in direct binding in all experiments, but M.Y⁺57-68 and N.335-349Y⁺ were more efficient competitors on HLA-A2 or B37 molecules, suggesting that they have a higher affinity for HLA molecules when in

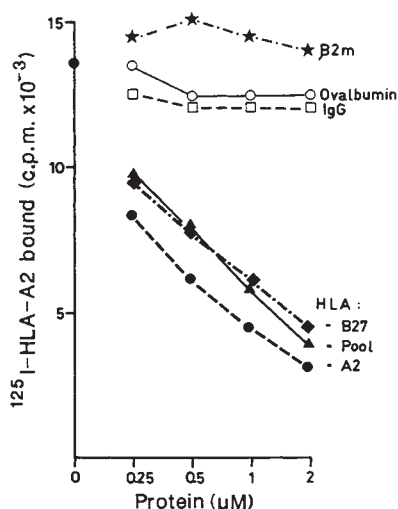
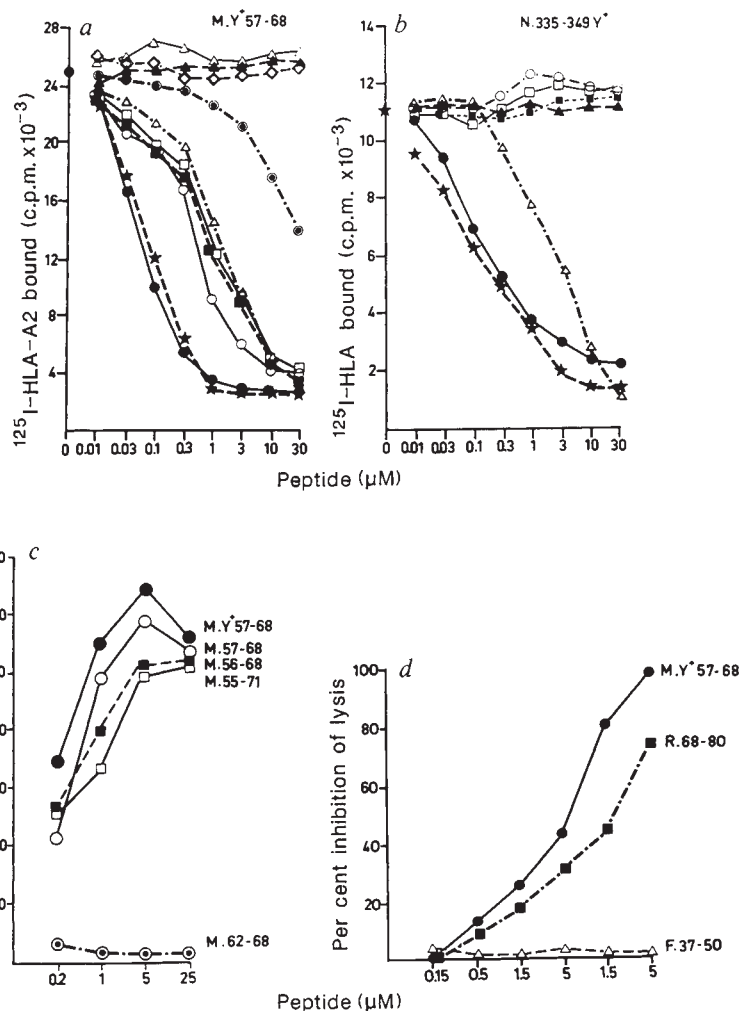


FIG. 2 Competition of various proteins with [¹²⁵I]HLA-A2 binding to M.Y⁺57-68. The data are expressed as the total amount of [¹²⁵I]HLA-A2 bound to the wells. The competitor proteins were: HLA-A2 (●—), HLA-B27 (◆—), HLA mixture obtained from various cells (▲—), human IgG (□—), β_2 -microglobulin (○—) and ovalbumin (○—). METHODS. Wells were coated with M.Y⁺57-68 as described in Fig. 1 and the indicated concentrations of unlabelled proteins were added. Incubation with [¹²⁵I]HLA-A2 (10⁵ c.p.m. 10⁻⁹ M) was for 20 h at 20 °C.

FIG. 3 Inhibition of [125 I]HLA binding to M.Y⁺57-68 and N.335-349Y⁺ by soluble peptides and correlation with functional tests. *a*, Inhibition of [125 I]HLA-A2 binding to M.Y⁺57-68. Competitor peptides were: M.Y⁺57-68 (●—), M.55-71 (□—), M.56-68 (■—), M.57-68 (○—), M.62-68 (●—), A2.170-185 (◇—), G.205-219C⁺ (▲—), F.37-50 (△—), N.335-349Y⁺ (★—) and G.265-279C⁺ (△—). *b*, Inhibition of [125 I]HLA-A2 binding to N.335-349Y⁺. Competitor peptides were: M.Y⁺57-68 (●—), N.335-349Y⁺ (★—), G.265-279C⁺ (△—), A2.139-159 (□—), N.147-158R⁺ (■—), G.255-269C⁺ (▲—), and F.37-50 (△—). *c*, Recognition of matrix peptides by anti-influenza CTL. The anti-influenza A Texas cell line from donor HLA-A2, A3; B7, Bw60 is specific for matrix peptides in association with HLA-A2. *d*, Inhibition of lysis by competitor peptides. The anti-influenza A Texas cell line from donor HLA-A2, A11; -B5, -B37 is specific for N.335-349Y⁺ in association with HLA-B37.

METHODS. *a, b*, Wells of microtitre plates were coated with 100 μ l of peptide M.Y⁺57-68 (5 μ g ml⁻¹) or peptide N.335-349Y⁺ (10 μ g ml⁻¹) as in Fig. 1. Competitor peptides at the indicated concentrations were preincubated for 2 h at 20 °C with [125 I]HLA-A2 (10⁻⁹ M) in PBS containing 1% albumin, 0.02% sodium azoture, 1 mM PMSF and 10 μ g ml⁻¹ trypsin inhibitor. The mixture was then incubated in microtitre plates for 20 h at 20 °C. After extensive washing the radioactivity in each well was counted. *c*, The effector cells were prepared as described²¹. The target cells were autologous Epstein-Barr virus-transformed B cells. Per cent specific lysis was measured after a 4 h incubation, using an effector: target ratio of 20:1, in the presence of matrix peptides at the concentrations shown. *d*, Competition experiments were performed as described²². Target cells were autologous EBV-transformed B cells, the inducer peptide was N.335-349Y⁺ at a final concentration of 5 μ g ml⁻¹ and the effector to target ratio was 4:1. Per cent specific lysis without competitor was 46%.



solution. The cross-inhibition found in competition assays was confirmed at the functional level. N.335-349Y⁺ inhibited the recognition of M.Y⁺57-68 by HLA-A2 restricted CTL (not shown) and M.Y⁺57-68 inhibited the recognition of N.335-349Y⁺ by HLA-B37 restricted CTL (Fig. 3d). Inhibition was also found with a strongly efficient peptide in direct binding, R.68-80, from the HIV-1 vpr protein.

It is likely that our method detects antigenic peptides binding to the antigen binding site because (1) peptides known to be targets for HLA restricted CTL bound, whereas control peptides did not; (2) a correlation was found between the ability of peptides derived from M.55-71 to sensitize target cells to CTL and their capacity to inhibit the binding of HLA molecules (these results have been extended to a series of 60 analogous peptides; manuscript in preparation); (3) in two different systems, peptides which inhibit the binding to a solid-phase peptide also inhibit its recognition by CTL; and (4) preliminary experiments with gag and nef proteins of HIV-1 show that the method detects two known T-cell epitopes of these proteins (to be published).

The observation that CTL target peptides bind to class I molecules that are not the restricting molecule stands in contrast to the earliest results in the class II system^{1,2}. Weak binding to class II molecules not known to function as antigen-presenting elements for the peptides concerned has been observed, however; some peptides have a broad range of reactivity with MHC class II molecules^{3,8}, and a single peptide is able to bind several HLA class II molecules at the cell surface (Rothbard, J.,

personal communication). Our experiments demonstrate a degree of preferential association of the matrix peptide for its restricting molecule HLA-A2, as well as a preferential association of HLA-B27 molecules for the B27-restricted peptide G.265-279C⁺, suggesting that quantitative differences in the peptide/HLA class I interactions may perhaps determine the pattern of MHC restriction observed. □

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- Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. R. *Nature* **317**, 359-361 (1985).
- Buus, S., Sette, A., Colon, S. M., Miles, C. & Grey, H. M. *Science* **235**, 1353-1358 (1987).
- Guillet, J. G. *et al Science* **235**, 865-870 (1987).
- Allen, P. M. *et al Nature* **327**, 713-715 (1987).
- Sette, A. *et al Nature* **328**, 395-399 (1987).
- Margalit, H. *et al J. Immunol.* **138**, 2213-2229 (1987).
- Rothbard, J. B. & Taylor, W. *EMBO J.* **7**, 93-100 (1988).
- Buus, S., Sette, A., Colon, S. M., & Grey, H. M. *Science* **242**, 1045-1047 (1988).
- Townsend, A. *et al Cell* **44**, 959-968 (1986).
- Gotch, F., Rothbard, J., Howland, K., Townsend, A. & McMichael, A. *Nature* **326**, 881-882 (1987).
- Maryanski, J. L., Abastado, J. P. & Kourilsky, P. *Nature* **330**, 660-662 (1987).
- Clayberger, C. *et al Nature* **330**, 763-765 (1987).
- Bodmer, H. C., Pemberton, R. M., Rothbard, J. B. & Askonas, B. A. *Cell* **52**, 253-258 (1988).
- Maryanski, J. L., Pala, P., Cerottini, J. C. & Corradin, G. *J. exp. Med.* **167**, 1391-1405 (1988).
- Chen, B. P. & Parham, P. *Nature* **337**, 743-745.
- Bjorkman, P. J. *et al Nature* **329**, 506-512 (1987).
- Bjorkman, P. J. *Nature* **329**, 512-518 (1987).
- Nixon, D. F. *et al Nature* **336**, 484-487 (1988).
- Walker, L. E. & Reisfeld, R. A., *J. Immunol. Meth.* **49**, R25 (1982).
- Bouillot, M., Choppin, J. & Levy, J. P. *J. Immunol. Meth.* **116**, 189-197 (1989).
- Healy, F. *et al Immunology* **141**, 2487-2496 (1988).
- Martinon, F. *et al J. Immunol.* (in the press).

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Location of high affinity Ca^{2+} -binding sites within the predicted transmembrane domain of the sarcoplasmic reticulum Ca^{2+} -ATPase

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CATION pumps bind and translocate ions with the intermediate formation of a phosphoenzyme. In spite of extensive knowledge of the primary and even secondary structures of several of these cation transport enzymes, however, no high affinity cation binding sites have yet been determined. Here we report the use of oligonucleotide-directed, site-specific mutagenesis to identify the amino acids involved in Ca^{2+} binding in one of these transport enzymes, the Ca^{2+} -ATPase of sarcoplasmic reticulum. Alteration of Glu 309, Glu 771, Asn 796, Thr 799, Asp 800 or Glu 908, each of which is predicted to lie near the centre of the transmembrane domain in putative transmembrane sequences M_4 , M_5 , M_6 and M_8 ¹⁻³ resulted in complete loss of Ca^{2+} transport function and of Ca^{2+} -dependent phosphorylation of the enzyme by ATP. Phosphorylation of each of the mutant enzymes with inorganic phosphate was observed, however, even in the presence of Ca^{2+} , which inhibits phosphorylation in the wild-type enzyme possessing an intact high affinity Ca^{2+} -binding site. These results suggest that at least six polar, oxygen-containing residues lying near the centre of the transmembrane domain provide ligands for one or both of the two high affinity Ca^{2+} binding sites in the Ca^{2+} -ATPase.

The binding of two Ca^{2+} ions to high affinity sites within the Ca^{2+} -ATPase molecule is an essential first step in Ca^{2+} translocation⁴. When these sites are occupied, ATP hydrolysis is triggered, the enzyme is phosphorylated, and a series of conformational changes in the enzyme results in the occlusion of Ca^{2+} and then to its translocation and release to luminal spaces of the sarcoplasmic reticulum⁴⁻⁷. To understand the mechanism of Ca^{2+} transport, it is essential to know where in the molecule Ca^{2+} is bound with high affinity. In our initial model of the structure and function of the Ca^{2+} -ATPase^{1,2}, we proposed that the high affinity Ca^{2+} binding sites were located in the highly negatively charged stalk domain. To test this hypothesis⁸, we

TABLE 1 Effect on Ca^{2+} transport of site specific mutations in the Ca^{2+} -ATPase

Nucleotide changes	Amino-acid changes	Calcium transport ($\mu\text{moles/min/mg}$ ATPase)
None	Wild-type	6.1
AAG-891 → ATG	Lys 297 → Met	<0.4
GAA-977 → CAG	Glu 309 → Gln	<0.1
GAG-2313 → CAA	Glu 771 → Gln	<0.1
GAG-2313 → GAC	Glu 771 → Asp	<0.1
ACC-2388 → GCC	Asn 796 → Ala	<0.1
ACG-2397 → GCA	Thr 799 → Ala	<0.1
GAC-2400 → AAT	Asp 800 → Asn	<0.1
GAG-2724 → GCG	Glu 908 → Ala	<0.1

Nucleotide changes were introduced into the cDNA for rabbit fast-twitch Ca^{2+} -ATPase by oligonucleotide-directed, site-specific mutagenesis using the method of Kunkel¹⁹. The altered cDNA in vector p91023(B) (ref. 20) was expressed transiently in COS-1 cells as described previously⁸. A microsomal fraction was prepared from the transfected COS-1 cells and tested for the presence of immunoreactive protein with antibody A52 (ref. 21) and Ca^{2+} transport as described previously^{3,8}.

demonstrated that COS-1 (monkey kidney) cells, transfected with full length rabbit fast-twitch Ca^{2+} -ATPase complementary DNA, express the protein with activity as much as 50-fold higher than that of the endogenous nonmuscle (kidney) isoform⁹ of the Ca^{2+} pump. We also demonstrated the feasibility of altering the cDNA encoding the Ca^{2+} -ATPase and of evaluating the effects of the mutations by analysing the overall and partial reactions of ATP-dependent Ca^{2+} transport. In another study³ we altered 30 Glx and Asx residues in the stalk sector, singly and in groups, and observed relatively little effect on Ca^{2+} transport. These observations led us to conclude that the high affinity Ca^{2+} -binding sites do not reside in the stalk region.

The transmembrane domain is also a candidate for the location of the high affinity Ca^{2+} binding sites. Of the 10 putative transmembrane helices, nine contain one or more charged residues over the length of 19–21 residues which would reside in the hydrophobic region of the bilayer (Fig. 1). The residues of most interest are glutamate, aspartate, glutamine, asparagine, serine and threonine, as their side chains often provide the oxygen ligands of high affinity Ca^{2+} -binding sites in Ca^{2+} -binding proteins¹⁰.

To test the hypothesis that residues in transmembrane sequences, which contain oxygen groups in their side chains, may be involved in high affinity Ca^{2+} binding, the mutations described in Fig. 1 were carried out. The full length cDNAs encoding these mutations were expressed transiently in COS-1 cells and microsomal fractions were assayed for Ca^{2+} -transport activity as described previously^{3,8}. Mutations to Glu 309, Glu 771, Asn 796, Thr 799, Asp 800 and Glu 908, each of which is predicted to lie near the centre of the transmembrane domain in

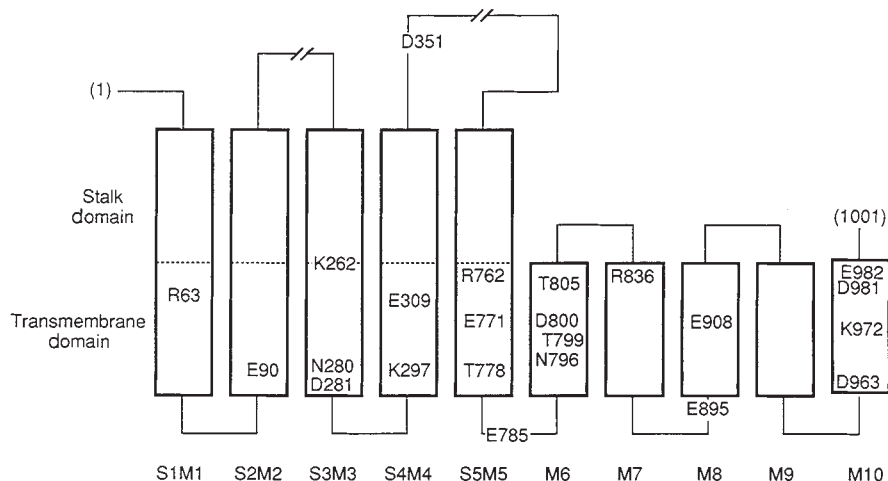


FIG. 1 Predicted distribution of the charged and polar amino acids, of the transmembrane region of the sarcoplasmic reticulum Ca^{2+} -ATPase altered in this study. The predicted positions of residues in M_3 and M_4 are modified³ from our earlier model² on the basis of comparisons with the hydrophathy plots of the amino-acid sequences of other cation-transporting ATPases. The position of the phosphorylated residue, Asp 351, is also indicated. The following mutations were made: Arg 63 → Leu; Glu 90 → Gln; Lys 262 → Met; Asn 280 → Ala; Asp 281 → Ala; Lys 297 → Met; Glu 309 → Gln; Arg 762 → Met; Glu 771 → Gln; Glu 771 → Asp; Thr 778 → Ala; Glu 785 → Ala; Asn 796 → Ala; Thr 799 → Ala; Asp 800 → Asn; Thr 805 → Ala; Arg 836 → Met; Glu 895 → Ala; Glu 908 → Gln; Asp 963 → Ala; Lys 972 → Met; Asp-Glu 982 → Ala-Ala.

TABLE 2 Conservation of Ca^{2+} -binding amino acids

Cation transport ATPase	Amino acid residue					
Rabbit SR Ca^{2+} ATPase ²	E309	E771	N796	T799	D800	E908
Rat PM Ca^{2+} -ATPase ¹⁴	E433	A866	N891	M894	D895	N977
Sheep kidney Na^+/K^+ ATPase ¹⁵	E327	E779	D804	T807	D808	V920
Rat gastric H^+/K^+ ATPase ¹⁶	E343	E795	E820	T823	D824	E936
Yeast H^+ -ATPase ¹⁷	V336	E703	A726	A729	D730	E803

Conservation among a series of cation transporting ATPases of amino acids proposed to be involved in high-affinity Ca^{2+} binding in the Ca^{2+} -ATPase of sarcoplasmic reticulum. The alignment is based on the proposals of N. M. Green, W. R. Taylor and D. H. MacLennan (unpublished review).

putative transmembrane sequence M_4 , M_5 , M_6 and M_8 , resulted in complete loss of Ca^{2+} -transport activity (Table 1). By contrast, the other mutations did not lead to any alteration in Ca^{2+} -transport function.

Because Ca^{2+} binding could not be measured directly due to the limited quantity of the expressed enzyme and to the high background in the crude microsomal fraction, we chose to assay the Ca^{2+} -dependent formation of a phosphoenzyme intermediate as a measure of Ca^{2+} binding to the enzyme. The addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to sarcoplasmic reticulum Ca^{2+} -ATPase, preincubated with Ca^{2+} , is followed by rapid transfer of ^{32}P to Asp 351 (see ref. 4 for a review). The assay serves as a measure of the intactness of the two high affinity Ca^{2+} binding sites, because both must be occupied for phosphoryl transfer from ATP to the aspartyl residue⁶. A phosphorylated intermediate was obtained in the presence of Ca^{2+} with the wild-type ATPase, but not with mutants in any of the six residues that are essential to Ca^{2+} transport, (Fig. 2a), consistent with the proposal that Ca^{2+} binding is deficient in these mutants.

A phosphorylated intermediate of the Ca^{2+} -ATPase can also be formed with inorganic phosphate (P_i), but only in the absence of calcium^{11,12}. Calcium binding seems to drive the enzyme into a conformation incompatible with phosphorylation by P_i . As shown in Fig. 2b, the wild-type protein and all of the transport-defective mutant proteins formed a phosphorylated intermediate with P_i in the absence of Ca^{2+} . Conversely, phosphorylation of the wild-type enzyme with P_i was completely inhibited in the presence of Ca^{2+} (Fig. 2c), whereas Ca^{2+} concentrations as high as 1 mM caused only a minor reduction in the level of the phosphorylated intermediate for five of the six mutants (Fig. 2c), demonstrating their inability to interact with Ca^{2+} . A phosphorylated intermediate was obtained with P_i in the presence of Ca^{2+} for the mutant Asn 796 $\rightarrow$ Ala, but at reduced levels, suggesting that Ca^{2+} binding was weakened.

These results are consistent with the hypothesis that one or both of the high affinity Ca^{2+} binding sites are disrupted or weakened in mutants in any of the six key residues and that the oxygen-containing side chains of Glu 309, Glu 771, Asn 796, Thr 799, Asp 800 and Glu 908 act as ligands for Ca^{2+} at the high affinity Ca^{2+} binding site. An alternative explanation for our observations is that the mutations result in structural alterations within the enzyme, preventing the E_2 to E_1 conformational change. This is unlikely, however, as three of the seven changes are isosteric (Glu to Gln; Asp to Asn), and thereby maintain size and most of the hydrogen bonding characteristics under the experimental conditions ($\text{pH} < 7.0$). The mutation, Glu 771 $\rightarrow$ Asp, affects side-chain length but not charge.

A third explanation is that by changing four acidic residues we have altered the charge balance within the hydrophobic sector of the enzyme. To evaluate this possibility, we altered all of the lysine and arginine residues within the transmembrane domain to neutral amino acids (Fig. 1). The mutant Lys 297 $\rightarrow$ Met (Table 1) exhibited greatly reduced Ca^{2+} -transport activity, whereas the others catalysed normal rates of Ca^{2+} transport. All of these mutants, including the Lys 297 $\rightarrow$ Met mutant, formed normal levels of phosphoenzyme in the presence of Ca^{2+} and ATP. Thus there is no reason to believe that Lys 297 might be

involved in Ca^{2+} binding. These observations make it unlikely that disruption of the internal compensation of negative and positive charges within the transmembrane domain is responsible for loss of Ca^{2+} -transport function.

On the basis of these results we propose that the high-affinity Ca^{2+} -binding sites are located near the centre of the transmembrane domain and that the carboxylate or carboxamide side chains of Glu 309, Glu 771, Asn 796, Asp 800 and Glu 908 and the hydroxyl group of Thr 799 act as ligands to form the high-affinity Ca^{2+} binding sites.

Kinetic experiments have demonstrated the presence of two interacting Ca^{2+} binding domains which are occupied sequentially by Ca^{2+} (ref. 5). We propose that occupancy of one Ca^{2+} binding site, formed by oxygen ligands from amino acids in different transmembrane segments, results in a reorientation of the segments, which increases the affinity of the second site for Ca^{2+} . As in previous models^{2,13} occupancy of both binding sites leads to conformational changes, which are transmitted to the nucleotide and phosphorylation domains to bring the bound ATP and Asp 351 into a reactive configuration. After the transfer of phosphate, further conformational motion utilizing the energy contained in the E_1P form of the enzyme, leads to a rotation or tilting of one or more of the transmembrane segments, disrupting the Ca^{2+} -binding domains and making the Ca^{2+} pair inaccessible from the external medium, but accessible to a channel leading to the luminal side of the membrane.

Other members of the family of cation pumps that form phosphoenzyme intermediates have recently been cloned and

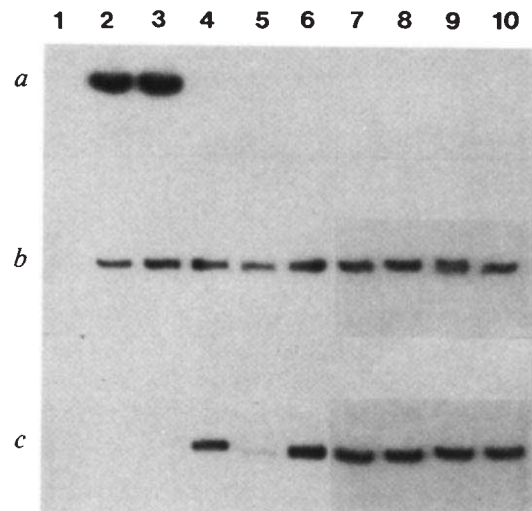


FIG. 2 Phosphorylation of the expressed Ca^{2+} -ATPase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (a), $^{32}\text{P}\text{P}_i$ in the absence (b) or presence (c) of 0.1 mM Ca^{2+} . Microsomes were prepared from COS-1 cells transfected with (1) vector alone, (2) wild-type, (3) Lys 297 $\rightarrow$ Met, (4) Glu 771 $\rightarrow$ Gln, (5) Asn 796 $\rightarrow$ Ala, (6) Thr 799 $\rightarrow$ Ala, (7) Glu 309 $\rightarrow$ Gln, (8) Glu 771 $\rightarrow$ Gln, (9) Asp 800 $\rightarrow$ Asn and (10) Glu 908 $\rightarrow$ Ala. Microsomes were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $^{32}\text{P}\text{P}_i$ as described below. A sample corresponding to 50 ng of Ca^{2+} -ATPase was applied to a 6% polyacrylamide gel for electrophoresis followed by autoradiography. The position of the phosphorylated protein corresponds to a relative molecular mass (M_r) of 110,000.

METHODS. The reaction mixture for phosphoenzyme formation with ATP in the presence of Ca^{2+} (a) contained 20 mM MOPS, pH 7, 80 mM KCl, 5 mM MgCl_2 , 0.1 mM CaCl_2 , 2 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and microsomal suspension corresponding to 0.2 μg of Ca^{2+} -ATPase. The reaction was carried out for 10 s in ice, and quenched with 7% (w/v) trichloroacetic acid/0.25 mM NaH_2PO_4 . The denatured protein was centrifuged, dissolved in lithium dodecyl sulfate and processed for electrophoresis and autoradiography. For the formation of a phosphoenzyme intermediate with P_i in the absence (b) and in the presence of Ca^{2+} (c), the reaction mixture contained 50 mM MOPS, pH 6.4, 10 mM MgCl_2 , 2 mM EGTA or 0.1 mM CaCl_2 , 0.5 mM $^{32}\text{P}\text{P}_i$, 20% (v/v) dimethyl sulfoxide and microsomal suspension corresponding to 0.2 μg of Ca^{2+} -ATPase. The reaction was carried out for 10 min at 25 $^\circ\text{C}$, and quenched with 0.25 M perchloric acid/4 mM NaH_2PO_4 . The denatured protein was separated on 6% polyacrylamide gels and exposed to X-ray film³.

sequenced¹⁴⁻¹⁷. Alignment of four of these with the amino-acid sequence of the rabbit sarcoplasmic reticulum Ca^{2+} -ATPase reveals a high degree of conservation of the six residues that we have identified in the Ca^{2+} -binding site (Table 2). The conservation of these residues indicates that the location and structure of the cation-binding sites may be similar for each of the different ion pumps, suggesting that some of these same residues may act as ligands for ion binding in each of the different ATPases. The translocation of Na^+ and K^+ or of one Ca^{2+} per cycle would follow a similar pathway, but with a slightly different cluster of co-ordinating atoms. Proton translocation could also occur in the same manner with complexation of hydronium ions with appropriately placed oxygens¹⁸. □

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1. MacLennan, D. H., Brandl, C. J., Korczak, B. & Green, N. M. *Nature* **316**, 696-700 (1985).
2. Brandl, C. J., Green, N. M., Korczak, B. & MacLennan, D. H. *Cell* **44**, 595-607 (1986).
3. Clarke, D. M. *et al.* *J. biol. Chem.* **264** (in the press).
4. Inesi, G. *A. Rev. Physiol.* **47**, 573-601 (1985).
5. Inesi, G. *J. biol. Chem.* **262**, 16338-16342 (1987).
6. Petithory, J. R. & Jencks, W. P. *Biochemistry* **27**, 5553-5564 (1988).
7. MacLennan, D. H. & Reithmeier, R. A. F. in *Structure and Function of Sarcoplasmic Reticulum* (eds S. Fleischer and Y. Tonomura) pp. 91-100 (Academic, London, 1985).
8. Maruyama, K. & MacLennan, D. H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3314-3318 (1988).
9. Lytton, J. & MacLennan, D. H. *J. biol. Chem.* **263**, 15024-15031 (1988).
10. Williams, R. J. P. *CIBA Fdn Symp.* **122**, 145-159 (1986).
11. Masuda, H. & de Meis, L. *Biochemistry* **12**, 4581-4585 (1973).
12. de Meis, L. & Masuda, H. *Biochemistry* **13**, 2057-2062 (1974).
13. Tanford, C., Reynolds, J. A. & Johnson, E. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7094-7098 (1987).
14. Shull, G. E. & Greib, J. *J. biol. Chem.* **263**, 8646-8657 (1988).
15. Shull, G. E., Schwartz, A. & Lingrel, J. B. *Nature* **316**, 691-695 (1985).
16. Shull, G. E. & Lingrel, J. B. *J. biol. Chem.* **261**, 16788-16791 (1986).
17. Serrano, R., Kielland-Brandt, M. C. & Fink, G. R. *Nature* **319**, 689-693 (1986).
18. Boyer, P. D. *Trends Biochem. Sci.* **13**, 5-7 (1988).
19. Kunkel, T. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 488-492 (1985).
20. Wong, G. G. *et al.* *Science* **228**, 810-815 (1985).
21. Zubrzycka-Gaarn, E., MacDonald, G., Phillips, L., Jorgensen, A. O. & MacLennan, D. H. *J. Bioenerg. Biomembr.* **16**, 442-464 (1984).

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Cell membranes impermeable to NH_3

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CLASSICALLY, there is a direct correlation between the lipophilic nature of a molecule and its rate of permeation across a biological membrane,^{1,2} so cell membranes should be more permeable to small, neutral molecules than they are to charged molecular species of similar size. Consequently, the distribution of NH_4^+ in biological systems is generally believed to be due to the rapid diffusion and equilibration of lipophilic NH_3 across cell membranes and the accumulation of NH_4^+ to be governed by pH differences between compartments. Here we report that renal tubule cells from the medullary thick ascending limb of Henle have an apical membrane which is not only virtually impermeable to NH_3 , but is also highly permeable to NH_4^+ . These remarkable properties have been incorporated into a model which explains how this renal epithelium can mediate vectorial movement of NH_4^+ between compartments of equal $\text{pH}^{3,4}$.

We monitored intracellular pH (pH_i) by measuring the fluorescence of 2,7-biscarboxyethyl-5(6)-carboxyfluorescein in two experimental systems derived from mouse kidney: the isolated perfused medullary thick ascending limb of Henle (IP-

MAL)^{5,6} and suspensions enriched in MAL tubules (S-MAL)⁷. Exposure of cells to NH_4Cl usually leads to intracellular alkalinization which is secondary to NH_3 entry^{8,9}. But addition of 20 mM NH_4Cl to the lumen of the IP-MAL (Fig. 1A, b) produces a striking decrease in pH_i (b-c), indicating that apical entry of NH_4^+ is much more rapid than for NH_3 . The pH_i reaches a nadir of 6.43 ± 0.08 (mean $\pm$ s.e.; $n = 7$) and does not recover over the ensuing 2 min (c-d). On withdrawal of luminal NH_4Cl (d), there is a rapid recovery of pH_i (d-e). In contrast to the effect of luminal NH_4Cl on pH_i , addition of NH_4Cl to the basolateral solution (f) leads to a rapid alkalinization (f-g): from 7.09 ± 0.08 to 7.40 ± 0.16 ; $n = 4$). Thus, the basolateral membrane is more permeable to NH_3 than it is to NH_4^+ , which is typical of other cell types examined so far⁹.

In S-MAL, addition of NH_4Cl in concentrations ≥ 0.5 mM leads to intracellular acidification whether tubules are buffered with HEPES (Fig. 1B) or with $\text{CO}_2/\text{HCO}_3^-$; the steady state pH_i values in HEPES-buffered medium were 7.04 ± 0.02 ($n = 11$) after addition of 20 mM NH_4Cl (b-e), 7.25 ± 0.02 ($n = 4$) after 0.5 mM NH_4Cl (b-d), and 7.40 ± 0.02 ($n = 3$) after 0.1 mM NH_4Cl (b-c). We obtained qualitatively similar results in IP-MAL with simultaneous addition of NH_4Cl to luminal and basolateral solutions. In addition, pretreatment with the stilbene anion transporter inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS; 0.2-0.5 mM), does not attenuate NH_4^+ -induced acidification (Fig. 1C), making bicarbonate exit an unlikely mediator of this acidification response. Therefore, NH_4^+ dominates over NH_3 entry across combined apical and basolateral membranes, which contrasts with results from other cell types.

In the MAL apical membranes contain both Ba^{2+} -sensitive K^+ channels and furosemide(bumetanide)-sensitive $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporters, whereas basolateral membranes contain ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)ATPase⁶. As the hydrated ionic radii of NH_4^+ and K^+ are almost identical¹⁰ and as NH_4^+ has been shown to be transported by these K^+ pathways^{3,4,11-15}, we determined whether NH_4^+ entry from lumen to cytosol was mediated by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter and/or the K^+ channels. Figure 2A shows that in S-MAL in the presence of K^+ transport inhibitors (bumetanide, BaCl_2 , and ouabain), addition of NH_4Cl (b) results in cell alkalinization ($\Delta\text{pH}_i = +0.26 \pm 0.01$; $n = 6$), which is consistent with NH_3 entry across basolateral membranes. As selective removal of Ba^{2+} (b-d) restores most of the acidification response (b-e), Ba^{2+} -sensitive K^+ channels seem to mediate most of the cellular NH_4^+ entry. In Fig. 2B, S-MAL are exposed to NH_4Cl in the presence of Ba^{2+} and furosemide (b-c), of Ba^{2+} alone (b-d), or in the absence of both (b-e). In the presence of Ba^{2+} , addition of furosemide further inhibits the acidification response, indicating that the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter mediates some of the cellular entry of NH_4^+ , in agreement with reported observations^{4,13}.

Figure 2C shows that addition of luminal Ba^{2+} and furosemide (b) in IP-MAL does not alter pH_i . But this combination markedly attenuates cell acidification on addition of luminal NH_4Cl (c). In the continued presence of luminal NH_4Cl , removal of barium and furosemide (d) leads to the expected intracellular acidification (d-e). These results confirm that apical NH_4^+ entry in the MAL is mediated by the combination of a Ba^{2+} -sensitive pathway and the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter. The absence of cellular alkalinization when apical NH_4^+ entry is reduced indicates that the NH_3 permeability of the apical membrane of the MAL must be very low. Thus, the low epithelial NH_3 permeability reported in the rat MAL⁴ seems to reside at the apical surface.

To determine whether exit of NH_4^+ in MAL can occur by its apical entry pathways, we assessed the effects of furosemide or Ba^{2+} on the recovery of pH_i after NH_4^+ withdrawal. As pH_i recovery following acidification in S-MAL is predominantly mediated by an amiloride-sensitive plasma membrane Na^+/H^+

exchanger⁷, we performed experiments with (Fig. 3A) and without Na^+ (Fig. 3B). Recovery of pH_i after NH_4^+ withdrawal is not altered by either Ba^{2+} or furosemide, even when Na^+/H^+ exchange, and hence the rate of pH_i recovery, is reduced by removal of extracellular Na^+ . Thus K^+ channels and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporters mediate the entry but not the exit of NH_4^+ : that is, these two transport pathways show apparent 'rectification' with respect to NH_4^+ transport.

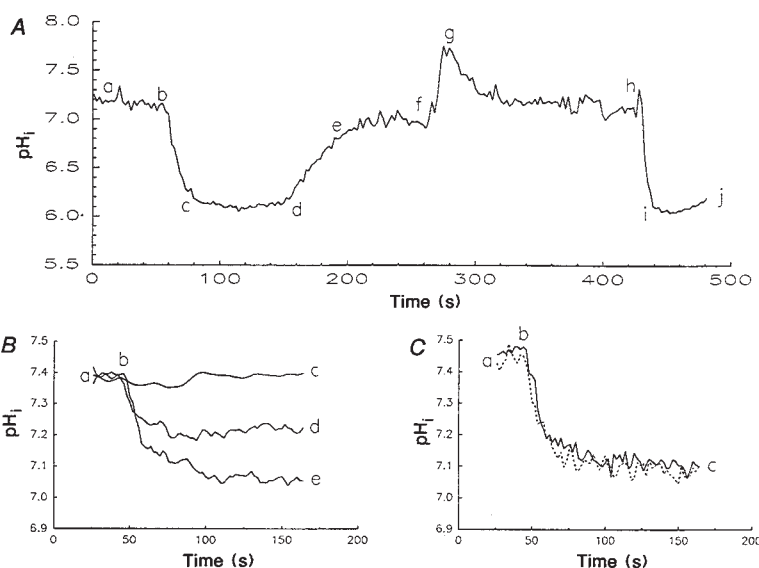
To localize Na^+/H^+ exchange activity to apical and/or basolateral domains of MAL plasma membranes, we undertook the

experiment shown in Fig. 3C. Addition of luminal NH_4Cl to the IP-MAL in the absence of luminal and basolateral Na^+ leads to acidification (*b-c*; pH_i falls to a steady-state value of 6.59 ± 0.09 (*c-d*); $n=3$). When ammonium is removed in the absence of extracellular Na^+ , pH_i does not recover (*d-e*), confirming that NH_4^+ does not leave using its entry pathways. Restoration of Na^+ to the apical (*f-g*) but not to the basolateral (*e-f*) medium gives prompt pH_i recovery. Thus, under our conditions, Na^+/H^+ exchange activity is located only on the apical membrane.

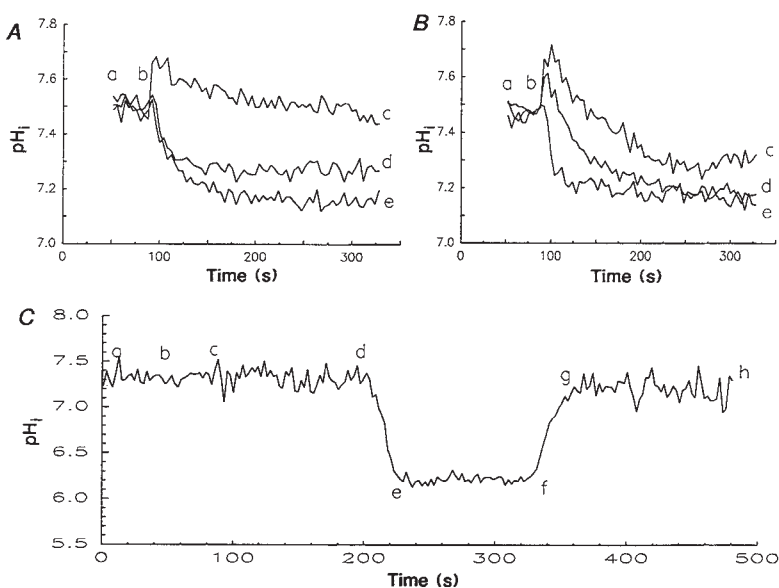
FIG. 1 A, Polarity of pH_i responses to NH_4Cl addition to either apical (perfusate) or basolateral (bath) solutions in the isolated perfused mouse medullary thick ascending limb of Henle. During time *a-b*, the IP-MAL is perfused and bathed in standard artificial medium (SAM). Steady state pH_i of IP-MAL in SAM was 7.26 ± 0.04 ($n=10$). Rapidly changing the perfusate to medium containing 20 mM NH_4Cl results in prompt acidification (*b-c*) to a steady-state pH_i of 6.2 (*c-d*). Changing the perfusate back to the NH_4Cl -free SAM (*d*) produces a rapid pH_i recovery (*d-e*). In contrast, addition of 20 mM NH_4Cl to the bath (*f*) leads to prompt alkalization (*f-g*), followed by pH_i recovery (*g-h*). Removal of NH_4Cl (*h*) gave prompt acidification to pH_i 6.2 (*h-i*), followed by pH_i recovery (*i-j*). B, Acidification of pH_i of MAL suspensions by addition of ammonium to medium bathing both apical and basolateral membranes. The resting pH_i in the S-MAL (*a-b*) was 7.43 ± 0.01 ($n=12$). Addition of NH_4Cl acidified pH_i at 500 μM (*b-d*) or 20 mM (*b-e*), but not at 100 μM (*b-c*). C, Insensitivity of ammonium-induced cell acidification to DIDS. At point *b*, 20 mM NH_4Cl was added to S-MAL either in the absence (solid line) or presence of 0.2 mM DIDS (dashed line). The pH_i dropped to 7.10 ± 0.03 and 7.08 ± 0.02 with and without DIDS (0.2–0.5 mM) respectively ($n=3$, paired $p=\text{NS}$).

METHODS. A, MAL nephron segments from CD_1 mice were isolated and perfused *in vitro*^{5,6}. Perfusate and bath SAM continued (mM): 140 NaCl, 5 KCl, 1.2 MgCl_2 , 5 L-alanine, 5.5 glucose, 1 CaCl_2 , and 3 HEPES: equilibrated with 100% O_2 , pH 7.4, at 37 °C. We used a computer-controlled inverted fluorescence microscope to monitor 2,7-bicarboxyethyl-5(6)-carboxyfluorescein (BCECF) fluorescence excitation ratios (495/440 nm, emission wavelength 530 nm). Background fluorescence was subtracted from fluorescence intensity at each excitation wavelength to obtain intensities of intracellular fluorescence. To express BCECF fluorescence ratios as pH_i , linear calibration curves ($r>0.97$) relating BCECF excitation ratios and extracellular pH were determined in medium containing 100 mM K^+ , 20 mM PO_4^{3-} , 20 mM HEPES, 12.5 mM Tris and 6.6–10 μM

FIG. 2 Role of barium- and furosemide (bumetanide)-sensitive pathways in NH_4^+ -induced acidification of MAL pH_i . A, Effect of barium on NH_4^+ entry in S-MAL. At point *b*, 20 mM NH_4^+ was added. In the absence of inhibitors (*b-e*), ammonium rapidly acidified pH_i . In the presence of bumetanide (1 mM), BaCl_2 (10 mM), and ouabain (5 mM) (*b-c*), ammonium resulted in rapid alkalization of pH_i ($\Delta\text{pH}_i=0.26 \pm 0.01$; $n=6$). But removal of only barium (*b-d*) resulted in restoration of a substantial portion of the acidification response to ammonium addition. The difference between curves *b-c* and *b-d* represents the component of NH_4^+ entry that is due to the Ba^{2+} -inhibitable pathway. B, Effect of furosemide on NH_4^+ entry in S-MAL. At point *b*, 20 mM NH_4^+ was added. As in A, the control acidification response is shown in (*b-e*). In the presence of furosemide (1 mM) and 10 mM BaCl_2 (*b-c*), cells initially alkalized then slowly acidified. Removal of furosemide (*b-d*) resulted in a faster rate of cell acidification, intermediate between the control rate (*b-e*) and that observed with Ba^{2+} and furosemide (*b-c*). Ouabain was omitted from these studies as MAL cells swell when exposed to ouabain and Ba^{2+} in the presence of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transport activity, that is, in the absence of furosemide¹⁷. C, Blockade of luminal NH_4^+ entry in IP-MAL by the combination of BaCl_2 and furosemide in the luminal solution. Segment *a-b* represents resting pH_i . Addition of BaCl_2 (10 mM) and furosemide (0.1 mM) to the luminal medium (*b*) did not alter pH_i . In the presence of barium and furosemide, acidification on addition of 20 mM luminal NH_4Cl (*c*) was markedly attenuated (*c-d*; $\Delta\text{pH}_i=-0.14 \pm 0.04$; $>80\%$ inhibition compared with *b-c* in Fig. 1A). But, pH_i fell promptly

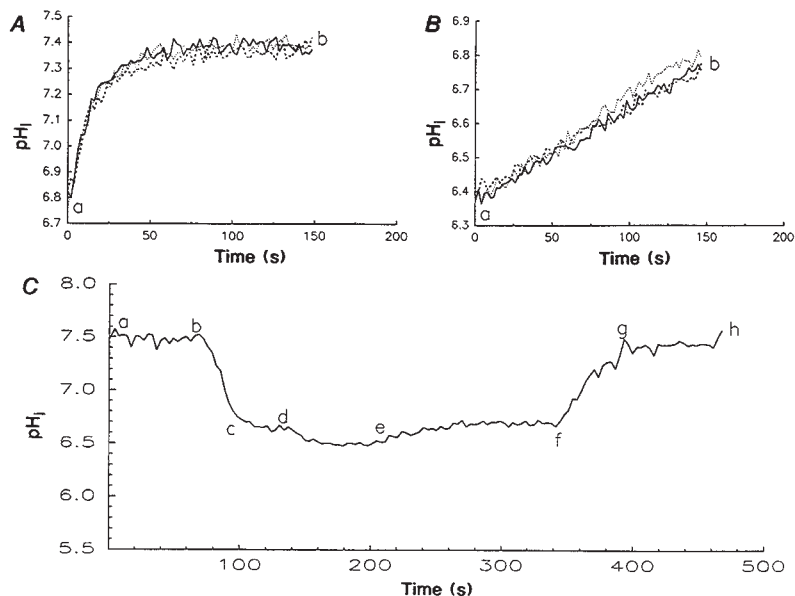


nigericin, pH 6–8¹⁶. Experiments were carried out without antidiuretic hormone and are representative of at least 3. B, S-MAL were isolated by serial collagenase digestion of CD_1 mouse outer medulla, consisted almost entirely of viable MAL tubules⁷ and were suspended in medium (S-SAM) at 37 °C containing (mM): 140 NaCl, 5 KCl, 2 NaH_2PO_4 , 1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, 20 mannitol, 0.6% dextran and 0.1% albumin, equilibrated with 100% O_2 , pH 7.4. BCECF fluorescence ratios were monitored at 500 nm/440 nm (emission 530 nm) in a computer-controlled spectrofluorometer and the background subtraction and calibration were as in A. No antidiuretic hormone was used and experiments are representative of at least 3.



(to 6.36 ± 0.09) with removal of both BaCl_2 and furosemide (*d*) in the continued presence of luminal NH_4Cl . Withdrawal of luminal NH_4Cl (*f*) led to rapid pH_i recovery (*f-g*) towards steady-state pH_i (*g-h*).

FIG. 3 Mechanism of NH_4^+ exit from MAL. **A**, Effect of furosemide or BaCl_2 on pH_i recovery of S-MAL incubated with 20 mM NH_4Cl for 10 min and then diluted in NH_4 -free S-SAM. Recovery of pH_i in S-SAM in the absence of inhibitors is shown by the solid line (a-b; initial pH_i recovery rate, 1.83 ± 0.10 pH unit per min; $n=5$). Neither furosemide (1 mM, dotted line) nor BaCl_2 (10 mM, dashed line) altered the rate of pH_i recovery. **B**, Inhibition of pH_i recovery from ammonium withdrawal in S-MAL in the absence of sodium: effect of furosemide or BaCl_2 . Conditions as in panel A, except that sodium in S-SAM was replaced with *N*-methyl-D-glucamine (NMG^+). Note that pH_i recovery was markedly attenuated in the absence of Na^+ (pH_i recovery rate, 0.20 ± 0.03 pH units per min; compare with panel A), and this response was unaffected by furosemide or Ba^{2+} . **C**, Localization of Na^+/H^+ exchange to apical membrane in IP-MAL. IP-MAL were perfused and bathed in SAM (a-b). At b, Na^+ was replaced in both solutions with NMG^+ and 20 mM NH_4^+ added to luminal medium. Removal of NH_4^+ (d) did not result in pH_i recovery. At e, replacement of NMG^+ in the bath with Na^+ and simultaneous addition of amiloride (0.1 mM) to the luminal fluid did not give significant pH_i recovery, but replacement of luminal NMG^+ with Na^+ , removal of luminal amiloride, and addition of basolateral amiloride (f) led to prompt pH_i recovery (f-g).



Our first and most significant observation is that a biological membrane can have a very low permeability to NH_3 . Given that NH_3 diffuses across lipid membranes by a solubility-diffusion process, the apparent low NH_3 permeability of the apical membrane of the MAL is probably due to the specific lipid composition of the bilayer. The virtually negligible water permeability of this membrane⁶ is also consistent with an unusual lipid structure-composition of the apical bilayer. But we do not know whether the specific alterations in the membrane that render it virtually impermeable to H_2O are responsible for the low NH_3 permeability. Our second observation is that a cell membrane can be rendered highly permeable to NH_4^+ by specific pathways that are usually considered to transport predominantly K^+ (for example, K^+ channels and the K^+ site on the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$

co-transporter). The third finding is that NH_4^+ transport through these cell membrane pathways shows rectified behaviour, permitting NH_4^+ entry into, but not exit from MAL cells. To our knowledge, rectification of NH_4^+ transport has not previously been described; the mechanisms involved are unknown.

Our results indicate that a novel mechanism for absorptive transport of ammonium ion could be operating to account for pH-independent accumulation of NH_4^+ in a specific tissue compartment, the interstitium of the renal medulla. In Fig. 4, ammonium ion enters the MAL cell from the luminal fluid through both K^+ channels (1) and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporters (2). Ammonium leak from the cytosol to the lumen must be low as the apical membrane has a low NH_3 permeability and as the apical NH_4^+ entry pathways do not mediate transport of ammonium ion from the cytosol to the lumen. Ammonium transport from the cytosol to the interstitium would be mediated by NH_3 diffusion across the basolateral membrane (3) associated with proton exit through the apical Na^+/H^+ exchanger (4). Protons entering the luminal fluid could be transported into the interstitium through the cation-permeable paracellular pathway (5). The apparent low permeability of the apical membrane to NH_3 would prevent the dissipation of ammonium gradients. This model suggests that competition between NH_4^+ and K^+ for the apical transport pathways in the MAL may partially explain the association between potassium balance and pH homeostasis. In addition, regulation of K^+ transport pathways in the MAL, other renal epithelia, liver and brain could have important effects on NH_4^+ metabolism. □

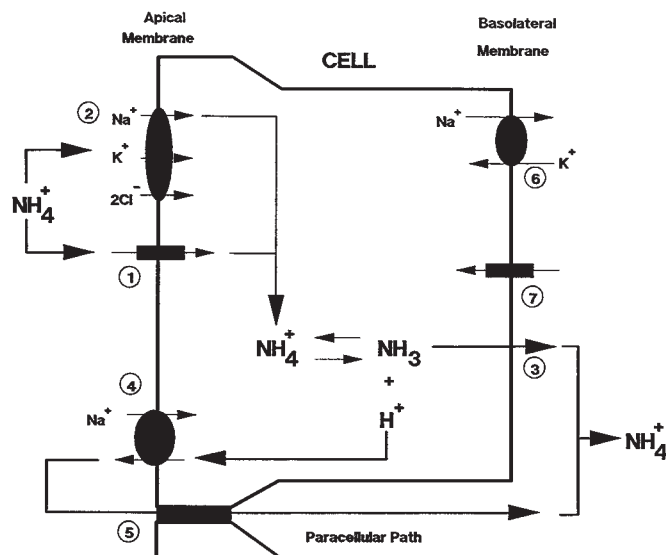


FIG. 4 Model for the pH-independent transport of ammonium. Pathways for $\text{NH}_4^+/\text{NH}_3$ transport are labelled 1-7: 1, the apical K^+ channel; 2, the apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter; 3, NH_3 diffusion across basolateral membrane; 4, the apical Na^+/H^+ exchanger; 5, H^+ diffusion through the paracellular pathway; 6, the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$; and 7, the basolateral K^+ channel. NH_4^+ may also recycle through the cell from interstitium to lumen by entering the cell on the K^+ -sites of basolateral transporters 6 and 7, and then exiting into the lumen by the H^+ site on the Na^+/H^+ exchanger. The magnitude of this back-flux must be less than the absorptive flux as net absorption has been documented in the MAL^{3,4}.

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- Overton, E. *Pflügers Arch. ges. Physiol.* **92**, 115-280 (1902).
- Collander, R. & Bärlund, H. *Acta bot. Fenn.* **11**, 1-114 (1933).
- Good, D. W., Knepper, M. A. & Burg, M. B. *Am. J. Physiol.* **247**, F35-F44 (1984).
- Knepper, M. A., Packer, R. & Good, D. W. *Physiol. Rev.* **69**, 179-249 (1989).
- Hebert, S. C., Culpepper, R. M. & Andreoli, T. E. *Am. J. Physiol.* **241**, F412-F431 (1981).
- Hebert, S. C. & Andreoli, T. E. *Am. J. Physiol.* **246**, F745-F756 (1984).
- Kikeri, D., Azar, S., Zeidel, M. L. & Hebert, S. C. *Clin. Res.* **36**, 521A (1988).
- Boron, W. F. & DeWeer, P. *J. gen. Physiol.* **67**, 91-112 (1976).
- Roos, A. & Boron, W. F. *Physiol. Rev.* **61**, 296-434 (1981).
- Kielland, J. *J. Am. chem. Soc.* **59**, 1675-1678 (1937).
- Zeiske, W. & Driessche, W. V. *J. Membrane Biol.* **76**, 57-72 (1983).
- Latorre, R. & Miller, C. *J. Membrane Biol.* **71**, 11-30 (1983).
- Kinne, R., Kinne-Safran, E., Schutz, H. & Scholermann, B. *J. Membrane Biol.* **94**, 279-284 (1986).
- Post, R. L. & Jolly, P. C. *Biochim. biophys. Acta* **25**, 118-128 (1957).
- Aldin, C. C. & Thomas, R. C. *J. Physiol. Lond.* **273**, 295-316 (1977).
- Thomas, J. A., Buchsbaum, R. N., Zimniak, A. & Racke, E. *Biochemistry* **18**, 2210-2218 (1979).
- Hebert, S. C. *Am. J. Physiol.* **250**, C920-C931 (1986).

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Formation of reverse rigor chevrons by myosin heads

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THE uniform angle and conformation of myosin subfragment 1 (S1) bound to actin filaments (F-actin) attest to the precise alignment and stereospecificity of the binding of these two contractile proteins^{1,2}. Because actin filaments are polar³, myosin heads must swing or rotate about the head-tail junction in order to bind. Electron microscopy of isolated thick filaments⁴ and of myosin molecules⁵ suggests that the molecules are flexible, but myosin fragments and crossbridges have been reported not to interact with inappropriately oriented actin filaments^{6,7}. Here we describe myofibrillar defects engendered by a site-directed mutation within the flight-muscle-specific actin gene of the fruitfly *Drosophila*. The mutation apparently retards sarcomere assembly: peripheral thick and thin filaments are misregistered and not incorporated into the Z-line. Therefore, a myosin filament encounters thin filaments with the 'wrong' polarity. We show that myosin heads tethered in a single thick filament can bind with opposite rigor crossbridge angles to flanking thin filaments, which are apparently of opposite polarities. Preservation of identical actomyosin interfaces requires that sets of heads originating from opposite sides of the thick filament swivel 180° relative to each other, implying that myosin crossbridges are as flexible as isolated molecules.

During an electron-microscope investigation of flight-muscle defects engendered by mutant *Drosophila* actin genes, we detected an unusual configuration of myosin crossbridges. One of the six actin genes of *Drosophila*, *Act88F*, is expressed only in the indirect flight muscles^{8,9}. We study effects of actin-gene

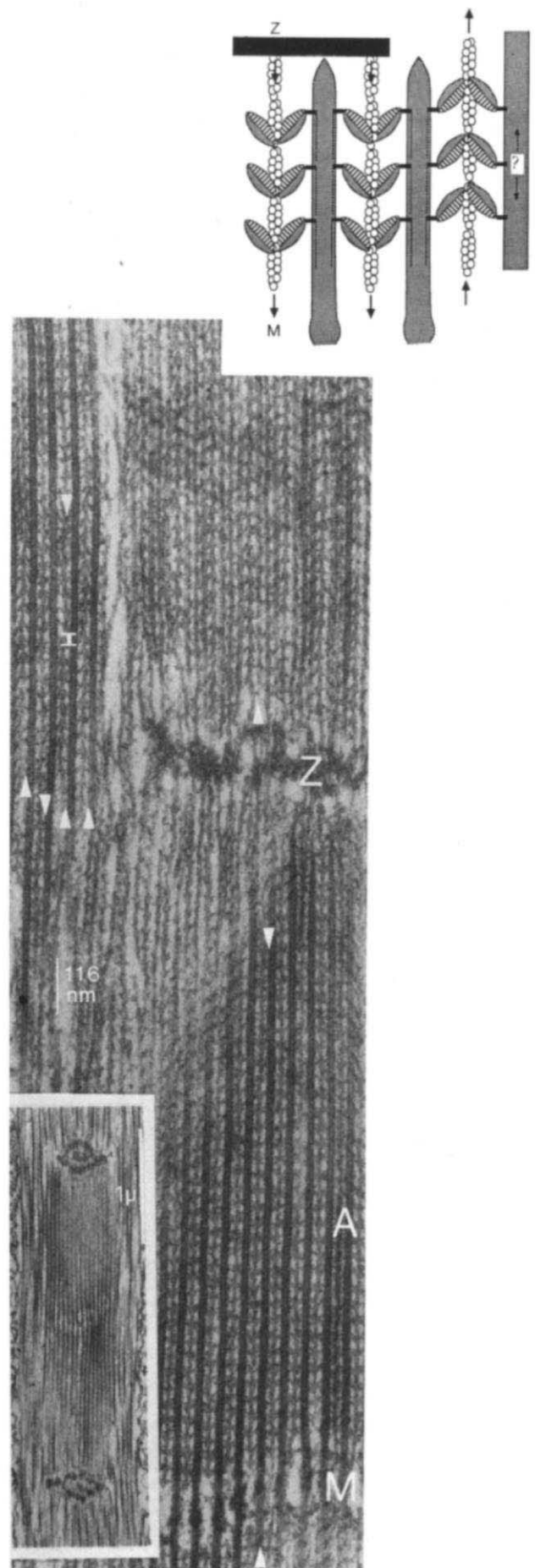


FIG. 1 Electron micrographs of 25-nm longitudinal sections of glycerinated flight muscle from *Act88F^{G6AA7T}* actin-gene mutant. Lower inset, one sarcomere at low magnification. Scale bar, 1 μ m. Z-disks are conspicuously abnormal. Whereas normal Z-disks are narrow and encompass the myofibril cross-section, mutant Z-disks are often partitioned into multiple layers, none of which extends across the fibril. Main figure, higher-magnification view of portion of a rigor sarcomere. Scale bar, 116 nm. The section includes only a single layer of myofilaments, either alternating thick and thin filaments or thin filaments only (top half-sarcomere). Filament polarity is indicated by black arrowheads. Uniform chevron polarity in central A-band (A) contrasts with polarity reversal along peripheral thin filaments. Note that polarity is uniform along entire length of a particular filament. Starred double bars mark apposed ends of apparently oppositely polarized peripheral filaments. M, M-line; Z, Z-line. Top inset, schematic diagram of reversed rigor chevrons and their relationship to sarcomere of *Act88F^{G6AA7T}* mutant. Grey bars, thick filaments; polymers of spherical actin monomers, thin filaments; small arrowheads, actin polarity. Three lateral repeats of thin filaments flanked by thick filaments are represented, two attached to the Z-disk (Z), one peripheral unincorporated and out of register. Three axial crossbridge repeats are shown. Ovals, myosin heads; actin-binding region stripes. Note that heads must swivel 180° around the S1/S2 junction to present same contact interface to actin. Myosin tail orientation is indicated by black lines over thick filaments; question mark, unknown tail polarity; chevrons, single myosin heads; M, M-line, Z, Z-line.

METHODS. Early embryos having the mutations *rosy*⁵⁰⁶ (brown eye colour) *Act88F^{KMB8}* (failure to produce actin in indirect flight muscles), and *ebony*^s (darkly pigmented cuticle) were transformed by injecting DNA cloned in a P-element vector¹⁹. Transforming DNA contained the marker allele *rosy*⁺ (red eye colour) and the *Act88F^{G6AA7T}* actin-gene mutation. The mutation was induced using oligonucleotide-directed mutagenesis²⁰, and sequence of mutant DNA verified using the chemical degradation method²¹. The transforming DNA was made homozygous by an appropriate series of crosses, and flight muscle inspected using electron microscopy.

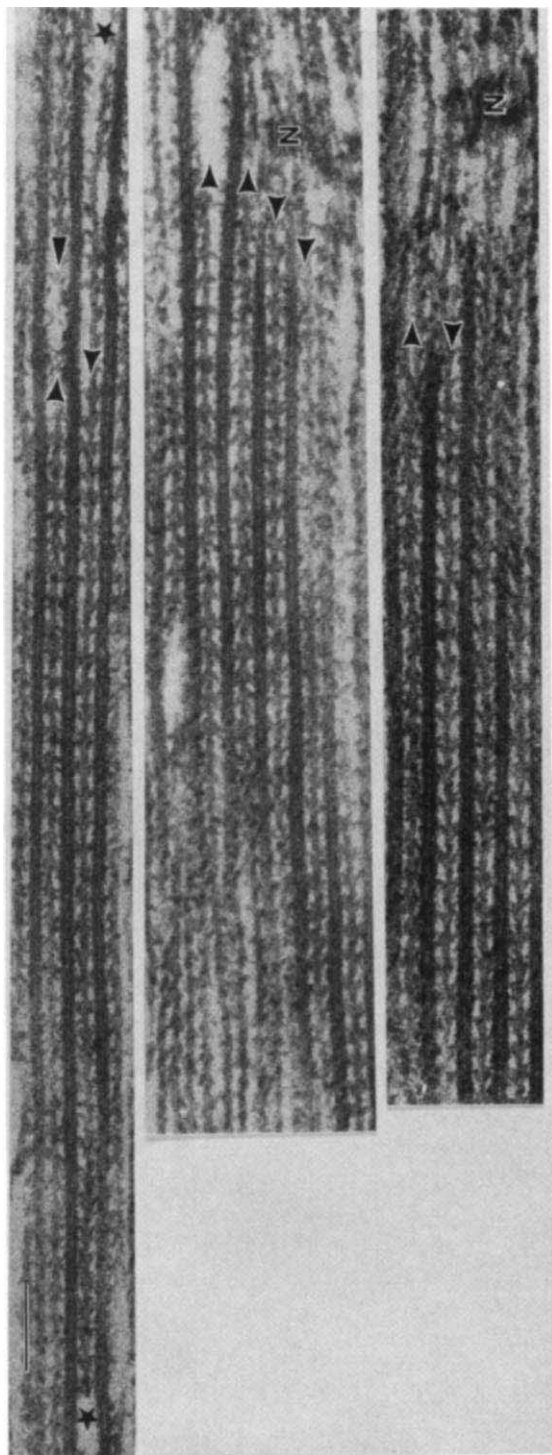


FIG. 2 Composite of electron micrographs of 25-nm longitudinal sections of peripheral regions of *G6AA7T* sarcomeres. Polarity of rigor chevrons along thin filaments is marked by appropriately oriented arrowheads. Chevrons are symmetrical about the thin filament and point in same direction along its full length, best demonstrated by filament between the stars in the left-hand panel. Note the reversed polarity of two thin filaments which approach one another in the same corridor; chevron reversal occurs within the same half of a myosin filament. Z, Z-band; Bar, 116 nm.

METHODS. Flight muscle was glycerinated *in situ* in the thorax and processed for electron microscopy according to ref. 10. Briefly, rigor was induced in fibres by washing out MgATP and fixing them in 3% glutaraldehyde (Tousimis), 0.2% tannic acid (Mallinckrodt) in 20 mM MOPS buffer, 5 mM EGTA, 10 mM MgCl_2 , 5 mM NaN_3 , pH 6.8, at 4 °C for 2 h, followed by 1% OsO_4 in 100 mM PO_4 , 10 mM MgCl_2 , pH 6.0, 4 °C, 30 min, block-stained in 2% aqueous uranyl acetate for 30 min, dehydrated, embedded in Araldite 506, and stained with 2% KMnO_4 and Sato lead stain.

mutations by mutagenizing *in vitro* the cloned *Act88F*, and subsequently introducing the altered gene into germline chromosomes of a strain whose resident *Act88F* gene has been eliminated by the nonsense mutation *KM88* (refs 10, 11). All actin in the flight muscles of the transformed flies is specified by the *in vitro* mutagenized allele, and any defects in myofibrillar structure and function can be correlated to properties of the altered actin.

The bottom inset of Fig. 1 shows a sarcomere from the flight muscle of one mutant, *Act88F^{G6AA7T}*, wherein amino acids six and seven, Gly-Ala, are converted to Ala-Thr. The dipeptide conversion renders the flies flightless. Observation of the *G6AA7T* homozygote mutant in the electron microscope shows that within the core of the myofibrils, bipolar thick filaments are precisely interdigitated with thin filaments. Most Z-lines, however, are elaborated into multiple levels, none of which crosses the entire myofibril diameter. At the periphery of each fibril, thick and thin filaments are not incorporated into the Z-line. As a consequence, highly ordered A-band regions are juxtaposed with variably registered peripheral thick and thin filaments.

Our evidence that *in situ* myosin heads are very flexible is based on crossbridge orientations in *Act88F^{G6AA7T}* flight muscles. Rigor crossbridges in indirect flight muscles of *Drosophila* appear similar to their more extensively studied counterparts in the waterbug *Lethocerus*^{12,13}. The regularity of this crossbridge lattice has suggested the operation of powerful constraints, possibly on crossbridge flexibility. In *Drosophila*, as in *Lethocerus*, myosin heads bind every 38.7 nm along thin filaments, forming in-register, angled chevrons which always point toward the M-line (M.C.R., unpublished observations). In central regions of *Act88F^{G6AA7T}*, we find in-register chevrons pointing towards the M-line (Fig. 1). In peripheral regions, chevrons point in either direction. At the border between the central and peripheral regions of the fibril, we frequently find thick filaments flanked by thin filaments apparently having opposite polarity (Figs 1, 2). All the rigor crossbridges attached to the more central thin filaments (anchored in the Z-line with correct polarity) point towards the M-line, whereas those bound to the more peripheral, unincorporated thin filaments point in the opposite direction (Figs 1, 2). That single thick filaments can form crossbridges with two oppositely oriented thin filaments demonstrates that actin-binding domains of the set of crossbridges along one side of the filament must be swiveled 180° relative to the set on the other side (Fig. 1, top inset).

Therefore, in agreement with earlier structural studies, our data shows that *in situ* crossbridges retain a high degree of torsional freedom. Under certain mechanical conditions, rigor crossbridges have been reported to reverse their angle of attachment to actin¹⁴. Electron-microscope observation of heads of isolated myosin molecules or of those tethered in thick filaments^{15,16}, and spectroscopy of myosin heads^{17,18} suggest considerable flexibility around the head-tail junction *in vitro*. Recent observations of the movement of actin filaments on substrate-bound myosin fragments (Toyoshima *et al.*, personal communication) suggest that when all heavy meromyosin (Hmm) are tethered in the same orientation, they are able to move actin filaments in either direction.

The work presented here represents the first synthesis of directed mutagenesis and muscle ultrastructure. We anticipate that in *Drosophila*, which lends itself to gene transfer and whose flight muscle allows detailed structural and functional analysis, this approach should identify the molecular interactions driving myofibrillar assembly and function. □

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1. Amos, L. A., Huxley, H. E., Holmes, K. C., Goody, R. S. & Taylor, K. A. *Nature* **299**, 467–469 (1982).
2. Milligan, R. A. & Flicker, P. F. *J. Cell. Biol.* **105**, 29–39 (1987).
3. Huxley, H. E. *J. molec. Biol.* **7**, 2381–308 (1963).
4. Knight, P. & Trinick, J. *J. molec. Biol.* **177**, 461–482 (1984).

5. Winkelmann, D. A. & Lowey, S. *J. molec. Biol.* **188**, 595-612 (1986).
6. Sheetz, M. & Spudis, J. *Nature* **303**, 31-35 (1983).
7. Trombitas, K. & Tigy-Seiges, A. *Nature* **309**, 168-170 (1984).
8. Fyberg, E. A. *Oxford Surv. Eukaryotic Genes* **1**, 61-86 (1984).
9. Ball, E. *et al. Cell* **51**, 221-228 (1987).
10. Hiromi, Y., Okamoto, H., Gehring, W. J. & Hotta, Y. *Cell* **44**, 293-301 (1986).
11. Okamoto, H. *et al. EMBO J.* **5**, 589-596 (1986).
12. Reedy, M. K. & Reedy, M. C. *J. molec. Biol.* **185**, 145-176 (1985).
13. Taylor, K. A., Reedy, M. C., Cordova, L. & Reedy, M. K. *Nature* **310**, 285-291 (1984).
14. Trombitas, K., Baatsen, P. & Pollack, G. *J. ultrastruct. molec. Str. Res.* **97**, 39-49 (1986).
15. Walker, M. & Trinick, J. *J. molec. Biol.* **192**, 661-667 (1986).
16. Walker, M. & Trinick, J. *J. Muscle Res. Cell Motil.* **9**, 359-366 (1988).
17. Mendelson, R. A., Morales, M. F. & Botts, J. *Biochemistry* **12**, 2250-2255 (1973).
18. Thomas, D. D., Seidel, J. C., Hyde, J. S. & Gergely, J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1729-1733 (1975).
19. Spradling, A. C. & Rubin, G. M. *Science* **218**, 341-347 (1982).
20. Nakamaye, K. L. & Eckstein, F. *Nucleic Acids Res.* **14**, 9679-9698.
21. Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499-460 (1980).

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Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process

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SUBTILISIN E, an alkaline serine protease consisting of a single polypeptide chain of 275 amino acids is produced from a pre-pro-protein¹. The pre-sequence functions as the signal peptide for protein secretion across the membrane². Deletion of the pro-sequence yields mature but inactive subtilisin³: the 77-amino acid pro-sequence must precede the mature subtilisin to guide the latter into an active conformation. Pro-subtilisin denatured in 6 M guanidine-HCl can be self-processed to the active enzyme intramolecularly, with concomitant cleavage of the pro-sequence, when dialysed against renaturing buffer⁴. We have constructed an active-centre mutant of pro-subtilisin (Asp 32 → Asn)³ which is not processed to active enzyme, unlike the wild-type pro-subtilisin, because intramolecular processing is prevented⁴. Here we report an intermolecular pathway for the refolding of the inactive mature protein to an active enzyme *in vitro* with the aid of exogenously added pro-sequence. We establish conditions under which the mature inactive form, as well as acid-denatured subtilisins Carlsberg and BPN⁵, can be renatured by the mutant pro-subtilisin.

Using a high expression secretion vector pIN-III-OmpA in *Escherichia coli*, we accumulated a large amount of mature, inactive subtilisin (S-pHT700; Fig. 1) by direct fusion of the OmpA signal peptide to the enzyme, which is then cleaved to mature subtilisin by signal peptidase I (ref. 3). We then dialysed S-pHT700 in 10 mM Tris HCl, pH 7.0, 6 M guanidine-HCl (henceforth called guanidine-HCl denaturant), against renaturing buffer (10 mM sodium phosphate, pH 7.1, 0.4 M (NH₄)₂SO₄) at 4 °C; aliquots were removed hourly and assayed for subtilisin activity at 37 °C (Fig. 2). No activity was detected, confirming our previous results⁴. When varying amounts of the pro-subtilisin mutant (proS-pHI216) in urea denaturant (50 mM Tris HCl, pH 8.1, 5 M urea) were mixed with S-pHT700 in guanidine-HCl denaturant and the mixture dialysed against renaturing buffer, we observed a time-dependent regain of activity (Fig. 2).

The amount of activity regained after three-hour dialysis is proportional to the concentration of proS-pHI216, indicating that it is a second-order kinetic process. We performed the renaturing experiments by dissolving S-pHT700 and proS-pHI216 in all permutations of urea and guanidine-HCl denaturants, then dialysed the mixtures against renaturing buffer. Optimal renaturation of S-pHT700 is achieved if it is first dissolved in guanidine-HCl denaturant. The denaturant used on proS-pHI216 does not affect the extent to which activity is regained, but for optimal renaturation, we incubated the mixture of denatured S-pHT700 and proS-pHI216 for as long as a week at -20 °C before dialysing against renaturing buffer. The molar ratio, *R*, of proS-pHI216 to S-pHT700 is important in the renaturation process: in a series of experiments we varied

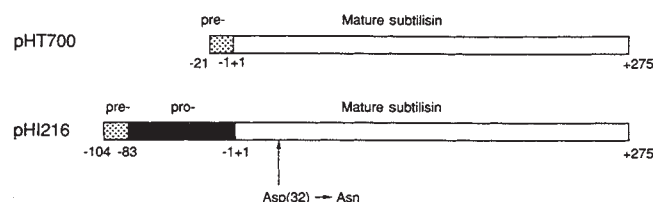


FIG. 1 Peptide products from plasmids pHT700 and pH216 (ref. 3). The empty box represents mature subtilisin, the solid box represents the pro-sequence and the shaded box represents the pre-sequence which is absent in our experiments, having been cleaved off by the signal peptidase. The pro-sequence of the pH216 pro-subtilisin mutant has six extra amino acids at the N-terminus compared with the pro-sequence of pro-subtilisin which consists of 77 residues³. In the mature subtilisin region of pH216 there is an amino-acid substitution at position 32 (Asp to Asn). This mutation was created by oligonucleotide-directed site-specific mutagenesis³. As Asp 32 is an essential part of the active centre, proS-pHI216 cannot be autoprocessed either *in vivo*³ or *in vitro*⁴ to produce active subtilisin.

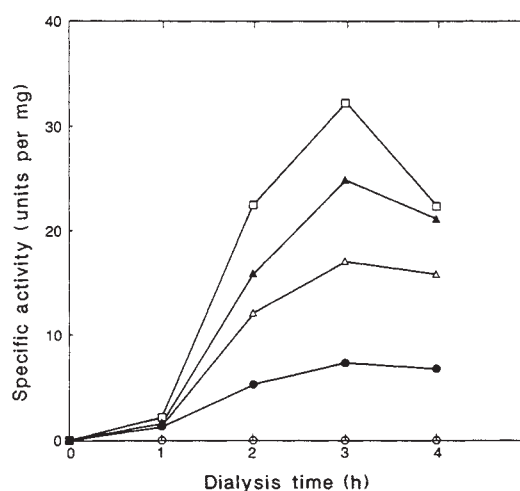


FIG. 2 Time course of activation of inactive subtilisin produced from pHT700 (S-pHT700) by proS-pHI216. Concentrations of proS-pHI216 solutions are: ○, 0.0; ●, 0.08; △, 0.16; ▲, 0.23 and □, 0.32 mg ml⁻¹.

METHODS. Purified S-pHT700 (20 μl, 0.3 mg ml⁻¹) in 10 mM Tris-HCl, pH 7.0, 6 M guanidine-HCl was mixed with 20 μl purified proS-pHI216 at different concentrations in 50 mM Tris-HCl, pH 8.1, 5 M urea, and dialysed against 30 ml 10 mM sodium phosphate, pH 7.1, 0.4 M (NH₄)₂SO₄, using the drop dialysis technique⁴. Aliquots removed at the times indicated were assayed for subtilisin activity at 37 °C using succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as substrate³. A unit of activity is defined as the amount of enzyme to produce 1 μmol *p*-nitroaniline per hour. Specific activities are calculated on the basis of the concentration of S-pHT700.

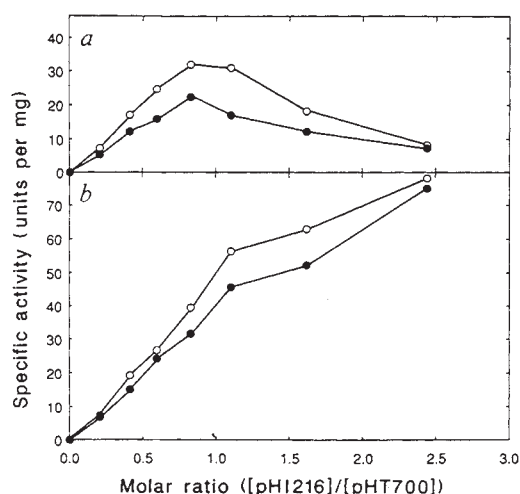


FIG. 3 Effect of concentration of proS-pHI216 on the activation of denatured S-pHT700. S-pHT700 (15 μ l, 0.3 mg ml⁻¹) in 10 mM Tris-HCl, pH 7.0, 6 M guanidine-HCl was mixed with 15 μ l proS-pHI216 in 50 mM Tris-HCl, pH 8.1, 5 M urea at the molar ratio indicated. Mixtures were dialysed for (●) two hours or (○) three hours against 25 ml 10 mM sodium phosphate, pH 7.1, 0.4 M (NH₄)₂SO₄ either *a*, immediately after mixing the two protein solutions, or *b*, after keeping the mixture at -20 °C for 7 d. Enzymatic activity was measured as described in the legend to Fig. 2.

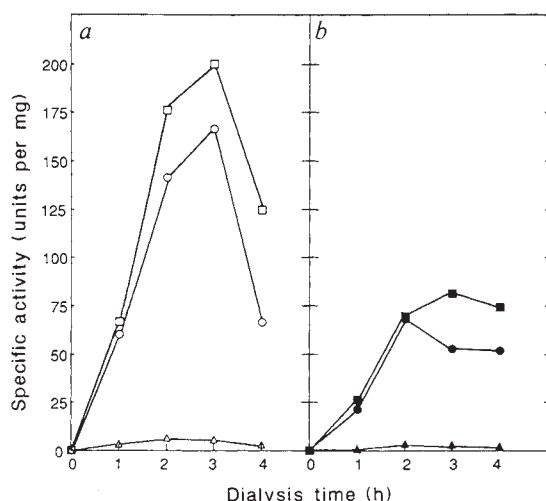


FIG. 4 Renaturation in the presence of the proS-pHI216 of acid-denatured subtilisins: *a*, subtilisin Carlsberg (Sigma) and *b*, subtilisin BPN' (Boehringer). Subtilisins in 50 mM citric acid, 10 mM boric acid, pH 2.2, at 0.3 mg ml⁻¹, were dialysed against 10 mM Tris-HCl, pH 7.0 6 M guanidine-HCl. The acid denatured subtilisin (15 μ l) was mixed with 15 μ l proS-pHI216 in 50 mM Tris-HCl, pH 8.1, 5 M urea, final pH 7-8. Concentrations of proS-pHI216 are: Δ and $\blacktriangle$, 0.0 mg ml⁻¹; $\circ$ and $\bullet$, 0.47 mg ml⁻¹; $\square$ and $\blacksquare$, 0.95 mg ml⁻¹. Mixtures were kept at -20 °C for 7 d and then dialysed against 30 ml sodium phosphate, pH 7.1, 2 mM CaCl₂, 0.5 M (NH₄)₂SO₄. Enzymatic activity was measured as described in legend to Fig. 2.

(Fig. 3), using different periods of preincubation in denaturant at -20 °C (mixtures remained liquid throughout), then dialysed for two or three hours against renaturing buffer. Comparison of Fig. 3*a* and *b* indicates that there could be at least two different modes of interaction between S-pHT700 and proS-pHI216. The first is seen after no preincubation with denaturant before dialysis (Fig. 3*a*): under these conditions of 'kinetic control', it seems that the denatured S-pHT700 cannot react with more than one proS-pHI216 ($R < 1$ for optimum renaturation). For $R > 1$, a 'high substrate inactivation' kinetic pattern is evident. When the mixture of denatured proteins is preincubated for a long time ('thermodynamic control'), as shown in

Fig. 3*b*, a much higher specific activity is ultimately attained (as high as 80 units per mg, ~20% of the activity of the native enzyme), and there is a monotonic increase in activity with increasing R value.

Like subtilisin E, Carlsberg and BPN' subtilisins cannot be renatured once they have been denatured. We therefore investigated whether proS-pHI216 could renature the denatured enzymes from these strains. Solutions containing 0.3 mg ml⁻¹ enzyme (denatured in 50 mM citric acid/10 mM boric acid, pH 2.2) were mixed with an equal volume of proS-pHI216 in urea denaturant and kept at -20 °C for 7 days before dialysing against renaturing buffer. As shown in Fig. 4 there is little renaturation in the absence of proS-pHI216. For $R = 2.4$, and with three hours dialysis, we recovered 2.3 and 5.5% respectively of specific activity for subtilisin Carlsberg and BPN'. The greater similarity of the pre-pro-subtilisin sequences of E (ref. 5) and BPN' (ref. 6), compared with Carlsberg⁷, together with some autolysis, probably accounts for these observations.

Our results demonstrate that the exogenously added proS-pHI216 can activate unfolded S-pHT700 and other subtilisins in an intermolecular process. Preliminary results indicate that this function can also be accomplished by a synthetic pro-sequence. Intermolecular complementation of protein folding by a pro-sequence, which is not a part of the active mature enzyme, is unprecedented in proteins consisting of a single polypeptide chain and which are devoid of disulphide linkages (in refs 8-10).

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- Boyer, H. W. & Carton, B. C. *Archs Biochem. Biophys.* **128**, 442-455 (1968).
- Wong, S. L. & Doi, R. H. *J. biol. Chem.* **261**, 10176-10181 (1986).
- Ikemura, H., Takagi, H. & Inouye, M. *J. biol. Chem.* **272**, 7859-7864 (1987).
- Ikemura, H. & Inouye, M. *J. biol. Chem.* **263**, 12959-12963 (1988).
- Stahl, M. L. & Ferrari, E. *J. Bact.* **158**, 411-418 (1984).
- Wells, J. A., Ferrari, E., Henner, D. J., Estell, D. A. & Chen, E. Y. *Nucleic Acids Res.* **11**, 7911-7925 (1983).
- Jacobs, W., Eliasson, M., Uhlen, M. & Flock, J.-L. *Nucleic Acids Res.* **13**, 8913-8926 (1985).
- Zabin, I. & Villarejo, M. R. *A. Rev. Biochem.* **44**, 295-313 (1975).
- Kim, P. S. & Baldwin, R. L. *A. Rev. Biochem.* **51**, 459-489 (1982).
- Goldenberg, D. R. *A. Rev. Biophys. biophys. Chem.* **17**, 481-507 (1988).

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Scanning tunnelling microscopy of Z-DNA

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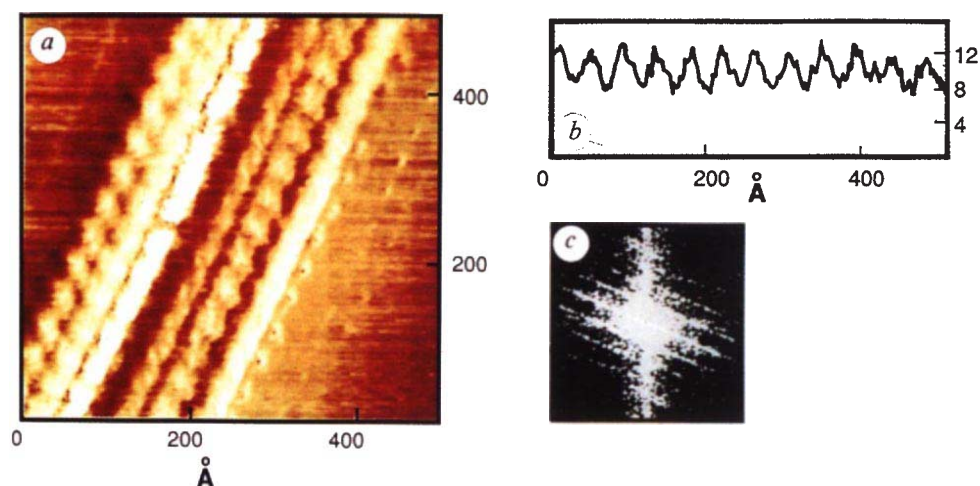
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SCANNING tunnelling microscopy (STM) has been used to map the surface topography of inorganic materials at the atomic level, and is potentially one of the most powerful techniques for probing biomolecular structure¹⁻³. Recent STM studies of calf thymus DNA^{4,5} and poly(rA) · poly(rU)⁵ have shown that the helical pitch and periodic alternation of major and minor grooves can be visualized and reliably measured. Here we present the first STM images of poly(dG-me⁵dC) · poly(dG-me⁵dC) in the Z-form. Both the general appearance of the fibres and measurements of helical parameters are in good agreement with models derived from X-ray diffraction⁶⁻⁸.

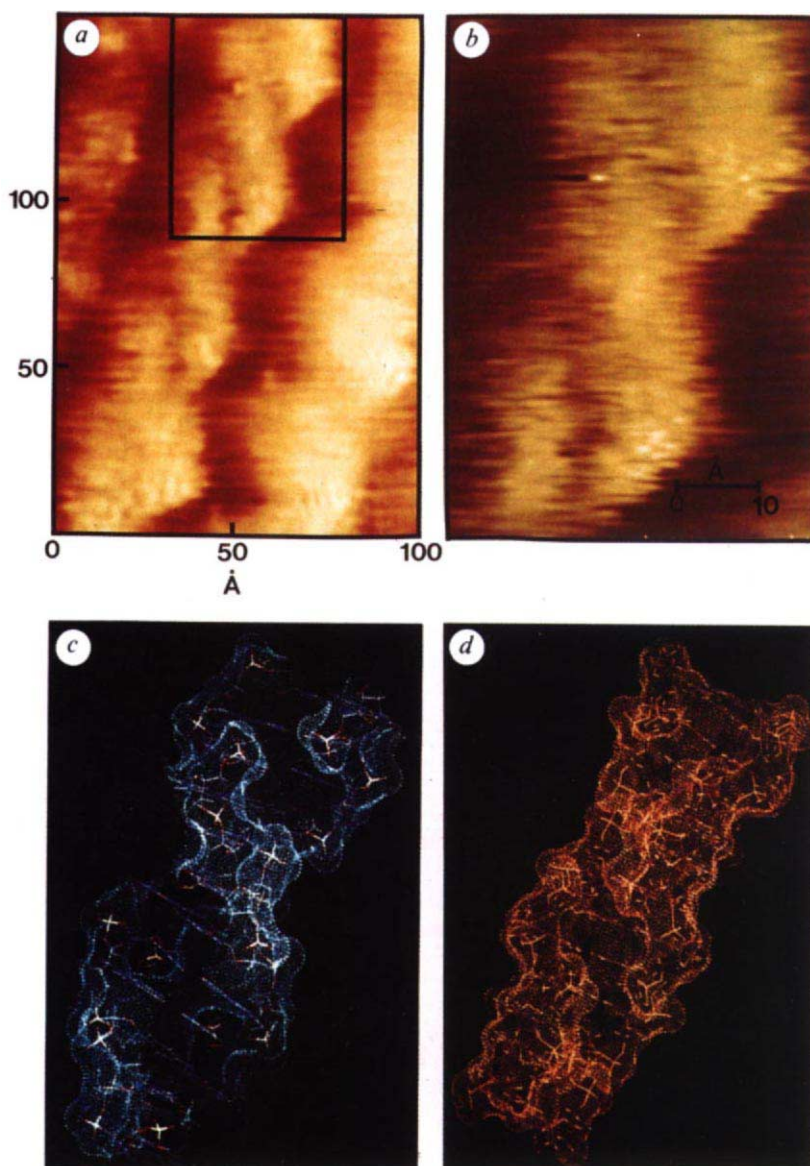
The characteristic left-handed twist of Z-DNA is clearly evident in all of the fibres of poly(dG-me⁵dC) · poly(dG-me⁵dC) shown in Fig. 1*a*. A plot of the vertical probe displacement

FIG. 1 Poly(dG-me⁵dC)·poly(dG-me⁵dC) fibres imaged by STM. Poly(dG-me⁵dC)·poly(dG-me⁵dC) (Pharmacia) was dissolved in 1 mM Na cacodylate, 10 mM NaCl, pH 7.4, at a concentration of $\sim 1.6 \text{ mg ml}^{-1}$. Concentrations were determined in a spectrophotometer (Beckman DU-8), using the standard $A_{260}=1$ for a solution containing $52 \mu\text{g ml}^{-1}$. One drop was deposited onto highly oriented pyrolytic graphite (Union Carbide) and dried for 15 min in a laminar flow hood. STM was performed in constant current mode, setpoint 0.93 nA, bias -90.3 mV , z range $49 \text{ \AA V}^{-1}$, using a Nanoscope II instrument equipped with a 9-mm scan head (Digital) and Pt:Ir(9:1) wire probe. Z -range roughness, after filtering to remove extraneous noise in the image, was $21.6 \pm 2.8 \text{ \AA}$. *a*, Colour-scale image of DNA fibres with left-handed twist. *b*, Profile of fibre number 1 (from left) showing the periodic change in vertical movement of the probe as a function of helical distance. Peaks are spaced $\sim 42 \text{ \AA}$ apart and differ in height by only $\sim 0.2 \text{ \AA}$. Of the six apparent fibres in the STM image, numbers 1, 2, 4 and 5 gave profiles sufficiently distinct



for quantitative analysis. Numbers 3 and 6 were blurred, and may in fact represent superposition of two molecules. *c*, Two-dimensional Fourier transform, showing strong equatorial lines at 41.5, 20.4, 14.5 and $\sim 7 \text{ \AA}$.

FIG. 2 STM image of poly(dG-me⁵dC)·poly(dG-me⁵dC) and molecular models. *a*, Overview of Z-DNA fibres. The sample was prepared as described in Fig. 1, except that it was overlaid with a drop of mineral oil to retard dehydration. STM was done in constant current mode, setpoint 0.75 nA, bias 132.8 mV , z range $50 \text{ \AA V}^{-1}$. *b*, Close-up of a single helical turn. *c*, Model of the electrostatic surface showing both major and minor grooves. Modelling was done on a SiliconGraphics Iris computer using the program 'Quanta' (Polygen). Coordinates for Z^{*}-DNA⁸ were obtained from the Brookhaven data base. Three copies of the tetranucleotide poly(dG-dC)₂ (file 1ZNA) were aligned to a dodecamer of Z₁(file 2ZNA) to determine their correct orientation. The segments were then linked by removing terminal O and H atoms between them and positioning P atoms by energy minimization. The electrostatic surface potential was calculated for a $1.7\text{-\AA}$ probe. Colours were scaled to enhance the contrast of positive (red) and negative (blue) areas. *d*, Model of the van der Waals surface.



versus distance along the main axis of fibre number 1 is shown in Fig. 1b. The slope of a plot of peak number versus distance traversed gives an average helical periodicity of 42.1 Å ($r^2 = 0.9996$, $n = 35$). This closely agrees with the value obtained by a two-dimensional Fourier transform of the image, as shown in Fig. 1c. The first strong diagonal corresponds to an axial period of 41.5 Å. This is somewhat smaller than the 45.4-Å repeat period of poly(dG-me⁵dC) · poly(dG-me⁵dC) determined by X-ray crystallography⁶, but is well within the range of Z conformers. For comparison, the repeat period of unmethylated poly(dG-dC) · poly(dG-dC) is 44.6 Å and for Z* it is as low as 43.2 Å (refs 7, 8).

Some shrinkage should be expected. The DNA samples were dried on graphite for about 15 min before the scan, and, in most cases, exposed to air for about 2 h during the scan. In our earlier STM study of nucleic acids⁵, helical periods of calf thymus DNA were ~7% less than the values for A- or B-DNA determined by fibre diffraction, and periods of poly(rA) · poly(rU) ~ 7% less than A-RNA. The present results with poly(dG-me⁵dC) · poly(dG-me⁵dC) are thus consistent with previous findings. Lang *et al.*⁹ have shown by electron microscopy that the contour length of double-stranded DNA and RNA decreases linearly with a logarithmic increase in the ionic strength of the stock solution. The helical repeat of RNA from 0.2 M NH₄Cl, for example, is 4–10% less than that found by crystallographic analysis. Although our samples were prepared in low-salt buffer, some of the shrinkage we observe may be attributable to increased ionic strength accompanying the drying process.

The average diameter of the fibres in Fig. 1a is 23.0 ± 3.7 Å ($n = 22$), whereas the crystallographic value is ~18 Å. Axial compression is apparently offset to some extent by an increase in thickness of the molecule. Cross-sectional profiles may also be slightly broadened for reasons having to do with the response time of the probe.

The fibres appear to be intact, with no unwinding or other sign of denaturation. The Fourier transform in Fig. 1c contains evidence of considerable substructure. In addition to the intensity line at 41.5 Å, which is produced by the crossover strand in the duplex, there are other lines which indicate periodic features at 20.4, 14.5 and ~7 Å. These are tentatively identified with the width of major and minor grooves¹⁰ and basic dinucleotide repeat unit.

Our results contrast with those of Beebe *et al.*⁴, whose STM images of calf thymus DNA on graphite were made under similar ionic conditions (10 mM KCl versus 10 mM NaCl). They observed stretching rather than shrinking, and ascribed deviations from standard dimensions to surface forces, dehydration and the possible intercalation of ions into the DNA duplex. Helical periods ranged from 27–50 Å in one micrograph to 46–52 Å in another, too wide a span to permit any conclusion as to the conformational state of the molecules. The highest values are too high for the most extended form of Z-DNA, and the 60-Å diameter quoted for the diameter of a single fibre is roughly three times that of any known form.

Figure 2a gives another region of poly(dG-me⁵dC) · poly(dG-me⁵dC) fibres with a close-up of a single turn of the helix in Fig. 2b. Using computer graphics, we have constructed molecular models to verify the Z-form and to assist in identifying specific features. The coordinates selected are for the Z* variant, which is observed in 0.2 M NaCl, in 50% EtOH, and in 0.7 M MgCl₂, conditions where water activity is low¹⁰. Our STM measurements indicate that the periodicity of poly(dG-me⁵dC) · poly(dG-me⁵dC) and Z* differ by only ~2 per cent, and we thus assume that the two molecules are dehydrated to about the same extent. We confirmed that the presence or absence of methyl groups on the cytosine bases does not significantly affect the geometry of the phosphate-ribose backbone⁶. A skeletal model of the Z*-DNA helix with the same orientation as the STM image is shown with two different surface representations in Fig. 2c and d. The first, a topographical map

of the electrostatic potential, seems to correspond to the molecular surface seen by the STM probe. Both major and minor grooves are visible, and their dimensions agree with our measurements of the STM image. The second surface, where atoms are drawn with van der Waals radii, does not correlate with the STM image. Only the minor groove is visible. Diffraction data show that the base pairs in Z-DNA are not positioned around the helical axis symmetrically. As a consequence, the C₅ atom in cytosine and the N₇ and C₈ atoms in guanine protrude into the major groove, completely filling the space and creating a convex instead of concave surface¹¹. These bases are apparently not detected by the STM probe.

It is becoming increasingly clear that seemingly small variations in molecular structure or electrostatic potential at specific sites can make a critical difference in how the nucleic acid is organized and how it is recognized by other molecules in the intracellular environment. Our results demonstrate that these variations can be distinguished by STM and that individual molecules can be examined in unprecedented detail. □

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1. Binnig, G. Rohrer, H., Gerber, C. & Weibel, E. *Phys. Rev. Lett.* **50**, 120–123 (1983).
2. Golovchenko, J. A. *Science* **232**, 42–47 (1986).
3. Hansma, P. K., Elings, V. B., Marti, O. & Bracker, C. E. *Science* **242**, 209–216 (1988).
4. Beebe, T. P., Jr *et al.* *Science* **243**, 370–372 (1989).
5. Lee, G., Arscott, P. G., Bloomfield, V. A. & Evans, D. F. *Science* **244**, 475–477 (1989).
6. Fujii, S., Wang, A. H.-J., van der Marel, G., van Boom, J. H. & Rich, A. *Nucleic Acids Res.* **10**, 7879–7892 (1982).
7. Behe, M. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1619–1623 (1981).
8. Drew, H., Takano, T., Tanaka, S., Itakura, K. & Dickerson, R. E. *Nature* **286**, 567–573 (1980).
9. Lang, D., Steely, H. T., Jr, Kao, C.-Y. & Ktistakis, N. T. *Biochim. biophys. Acta* **910**, 271–281 (1987).
10. Pullman, A. & Pullman, B. *Q. Rev. Biophys.* **14**, 289–380 (1981).
11. Saenger, W. *Principles of Nucleic Acid Structure* (Springer, New York, 1984).

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Figure legends must not exceed 300 words and ideally should be much shorter, and should use telegraphic form wherever possible. The figure should be described first, then, briefly, the method. Reference to a method described elsewhere is preferable to a full description. Methods should not be described in detail in the text.

References should be numbered sequentially as they appear in the text, followed by those in tables and finally those in the figure legends. Reference numbers apply only to papers published or in the press, and a different number should be given to each paper cited. All other forms of reference (including unrefereed abstracts) should be included in the text as personal communication, manuscript in preparation or preprint (with number and institution where appropriate). Text should not be included in the reference list. References should be abbreviated according to the *World List of Scientific Periodicals* (fourth edition, Butterworth, London, 1963–65). The first and last page numbers should be cited. Reference to books should clearly indicate the publisher and the date and place of publication.

Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided as much as possible and, when used, defined. Editors will shorten words if necessary. In case of doubt, SI units should be used.

Footnotes should not be used except for changed addresses or to identify the corresponding author if different from the first-named.

Acknowledgements should be brief. Grant numbers and contribution numbers are not allowed.

Mycoplasma infection of cultured cells

R.J. Hay, M.L. Macy and T.R. Chen

Mycoplasma contamination is tough to detect and even more difficult to eradicate. It is best to start over fresh from clean cell stocks, but several elimination options are available.

OVER the past three decades mycoplasmal infection, which is capable of drastically altering the structure and function of host cells, has been a common occurrence in cell cultures. Mycoplasma and Acholeplasma of the Order Mycoplasmatales are among the smallest living prokaryotes (0.2–2 µm in diameter) and lack a cell wall. Their circular double-stranded DNA is comparatively rich in adenine and thymine, and 16S and 23S ribosomal RNAs are present in their cytoplasm. Between 5 and 35 per cent of cell cultures in current use are infected with mycoplasmas, with the species *Mycoplasma orale*, *M. hyorhinis*, *M. arginini* and *Acholeplasma laidlawii* representing the majority of contaminating isolates^{1,2}.

Many species of mycoplasma exert no cytopathogenic effects on host cells, making infection difficult to detect. In cell culture the concentration of mycoplasma can easily reach 10⁷–10⁸ colony forming units per millilitre without overt turbidity. Coupled with the fact that most mycoplasmas are resistant to commonly used antibiotics, these properties render mycoplasmas an interesting but formidable foe for cell culture scientists.

Detection methods

Numerous methods for detecting mycoplasma infection have been developed², but the most sensitive and specific is the standard microbiological cultivation procedure. Before use in such direct culture tests, new lots of horse serum, yeast extract, and other ingredients should be pretested for their growth-promoting properties and absence of toxicity. Anaerobic incubation (in 95 per cent nitrogen, 5 per cent CO₂) dramatically increases the sensitivity.

Both the direct culture test and a sensitive DNA staining reaction employing either the bisbenzimidazole fluorochrome Hoechst 33258 (ref. 3) (Fig. 1) or the DAPI (4'-6-diamidino-2'-phenylindole)⁴ reagent are used routinely in many laboratories to check incoming cell lines and working cell stocks. Because some strains of *M. hyorhinis* isolated from cell cultures are difficult to cultivate in artificial media, quality-control programmes should include at least one other test in addition to standard microbial cultivation in broth and agar. For any test, positive controls consisting of *M. arginini*, *M. orale*, and *A. laidlawii* are recommended. Many laboratories have modified the fluorescent DNA stain of Chen³ by incor-

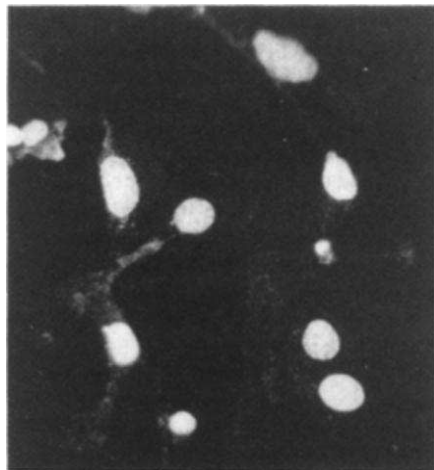


FIG. 1 Fluorescence photomicrograph of Vero indicator cells infected with mycoplasma and stained with Hoechst 33258. The large oval fluorescing bodies are Vero nuclei and the mycoplasma are seen as numerous points of light in the background.

porating an indicator cell line (usually 3T6 — ATCC.CCL 96 mouse embryo, or Vero — ATCC.CCL 81 monkey kidney)⁵.

Biochemical methods for detecting mycoplasma include the measurement of arginine deiminase, hypoxanthine phosphoribosyl transferase migration rates, uridine to uracil incorporation ratios, and assays of uridine or adenosine phosphorylase activities. Another interesting detection method, used especially in laboratories lacking a fluorescence microscope, involves a biological assay utilizing 6-methyl purine deoxyriboside (6-MPDR). This non-toxic analogue of adenosine is converted by adenosine phosphorylase into 6-methyl purine and 6-methyl purine riboside, both of which are toxic to mammalian cells. Mycoplasma generally have much higher levels of adenosine phosphorylase than do cultured mammalian cells, and accordingly hydrolyse 6-MPDR more rapidly. The release of toxic products from 6-MPDR in the presence of mycoplasma is detected by including a sensitive indicator cell line, such as 3T6, in the test system⁶ (Life Technologies, Inc., Grand Island, New York, USA).

But many biochemical detection methods give inconsistent results when comparing different cell lines, and have lower sensitivities than the cultivation and Hoechst staining methods, making their use problematic². Recently, a nucleic acid hybridization assay for mycoplasma has been developed that has a sensitivity and reliability approaching that of the Hoechst stain. The assay uses a tritium-labelled,

single-stranded DNA probe complementary to the ribosomal RNA of mycoplasma and acholeplasma to detect the contaminants specifically. Reagents for the test are available in a kit from Gen-Probe, Inc., San Diego, California, USA.

Controlling contamination

Mycoplasma infection of cell cultures may result from contact with other previously infected cultures, or perhaps less commonly, may be transferred through the cell culture technician or contaminated reagents. It is often helpful to identify the species of mycoplasma present to narrow down the most probable source of contamination^{2,5,7}. Mycoplasmas from human, bovine, and porcine sources account for the majority of the contaminations observed. The adoption of appropriate handling and testing procedures minimizes the risk of contamination from these sources. As in all quality control work with cultures of unknown infectivity, it is prudent to use a vertical laminar-flow biohazard hood — preferably one that is isolated from the other standard cell culture facilities. Technicians should continually be aware of the danger of contaminating clean cultures with aerosols from mycoplasma-containing cultures manipulated in the same area. The inner surfaces of the hood should be disinfected with 70 per cent ethanol or 10 per cent bleach solutions between uses, and mouth pipetting should be prohibited.

It has been common practice to filter cell culture media and reagents through 0.2 µm-pore filters in the final stages of preparation. While single or series filtration steps at this level usually suffice, it is important to emphasize that the smallest mycoplasmas may be forced through such filters at higher pressures (greater than 15 pounds per square inch). Some suppliers of serum for cell culture are now using 0.1 µm filters to overcome this problem.

The indiscriminate use of antibiotics in cell culture fluids permits repeated lapses in aseptic technique. Consequently, the system may become contaminated with resistant organisms, especially mycoplasmas. To avoid this, antibiotics should only be used in special applications and for short durations.

It is essential for managers of cell culture facilities to establish a routine screening programme for all forms of microbial contamination, including mycoplasmas. Ideally, seed stocks of each cell line in frequent use should be cryopreserved in

liquid nitrogen and thoroughly tested before use. Such stocks then serve as a ready reserve of characterized contaminant-free material for the subsequent production of working cell stocks⁸.

Eliminating infection

Four general procedures have been used to eliminate mycoplasmas from infected cell cultures: treatment with hyperimmune sera⁹; passage through mice, or exposure to murine macrophages^{10,11}; successive incubation of contaminated cells in 5-bromouracil and Hoechst 33258, followed by exposure to light¹²; and selective killing with antibiotics or other drugs. The use of antibiotic mixtures is by far the most common method, but it is time-consuming and labour-intensive, and may yield varying degrees of success. Antibiotics may also select for a cell population differing from the original cell line.

Antibiotic treatment can be most effective if the application is based on antibiotic sensitivity tests. Tylosin has been used successfully in combination with spectinomycin against *M. hyorhinis* contamination of the human colon adenocarcinoma cell line LoVo, CCL 229 (ref. 13). Tiamuline, minocyclin and other tetracyclines have been effective in other cases¹⁴. Several proprietary antibiotic mixtures for eliminating mycoplasmas from cell culture are now on the market. Some have been successful, but toxic conditions have been reported, even at low dosages (1.4 $\mu\text{l ml}^{-1}$).

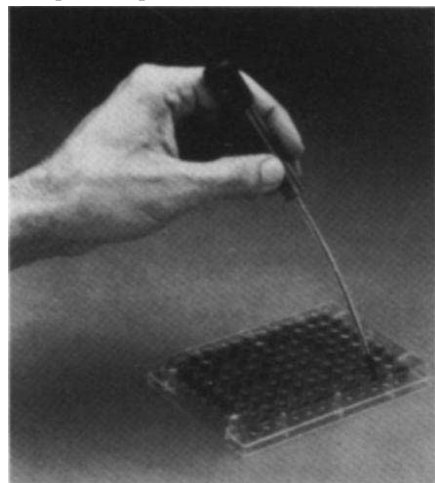
Attempts to eliminate mycoplasmas from contaminated cells should be considered only as a last resort. It is often far better to eliminate the problem completely by autoclaving the infected cultures and replacing them with fresh stocks known to be mycoplasma-free. □

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Tissue culture trends

Products for culturing cells and tissues will be on display at next week's annual meeting of the Tissue Culture Association in Orlando, Florida.

TAKING samples from very small amounts of liquid is now possible with C.P. Instrument's Micro pH Electrode (*Reader Service No. 101*). The **microelectrode** is small enough to measure pH in microwell plates, serum cups and capillary tubes. The instrument is a combination microprobe/electrode with a tip diameter of less than 1000 μm . The flat sensor tip allows measurements as small as 10 μl to be taken on filter paper or agar gel. The company says the unit is most accurate in the pH range of 3-9, and that its low



The C.P. Instruments microelectrode reaches into small spaces. output impedance eliminates noise interference and provides stable readings within a temperature range of 0-75 °C.

Accurate Chemical is announcing the availability of a wide range of Biocarb **gangliosides** to be used as antigenic blood group determinants and as receptors for toxins, hormones and viruses (*Reader Service No. 102*). Gangliosides can be used as membrane modulators of malignant processes in cell membranes and as pharmacologic agents in the treatment of neuronal lesions. Accurate says gangliosides have been shown to have important roles in the synaptic transmission of nerve impulses, as growth factors, and as sensitive cell surface regulators in various CNS diseases like Alzheimer's disease. Most gangliosides are available in 0.1-1 mg quantities for \$48-100 (US).

Dynatech recently introduced its Artek Omnicon 3600 **image analyser** (*Reader Service No. 103*). The micro-computer system possesses minicomputer power and is capable of enlarging and displaying a section of sample on its high resolution monitor screen. The company says this



Dynatech's Omnicon 3600 image analyser. enables selected measurements to be made from more than 20 parameters. Typical applications include cell or tissue analysis and cancer assays, and the basic Omnicon unit sells for \$31,790 (US).

Conductive environments

Newly available from Collaborative Research is a line of human leukocyte monoclonal antibody-coated Biomag particles that provide an alternative to FACS analysis and bulk sorting (*Reader Service No. 104*). The **magnetic cell-sorting particles** are biodegradable, stable in a wide variety of solvents, and available in 2-and 10-ml sizes (\$65 (US) and \$210 (US), respectively). For more information regarding Collaborative Research's list of reagents, consult their 1989 product catalog now available.

Sigma Cell Culture is introducing colostrum-free **neo-natal calf serum** to its line of sera for cell and tissue culture (*Reader Service No. 105*). The serum is collected from bovine calves during the post-natal period, less than 24 hours after birth and before nourishment. The animals are certified as free of antibiotics and in good health, says Sigma, and stomach contents are checked for signs of nursing. The company says blood chemistries of the neo-natal serum are similar to those of fetal bovine serum, and that IgG levels in the sera are low. Sigma finds colostrum-free neo-natal serum (\$32.50 (US) for 100 ml) to be the logical alternative to fetal bovine serum.

Boehringer Mannheim has a new cytokine — **human recombinant interleukin 6** (*Reader Service No. 106*). As a growth factor for murine and human B-cell hybridomas, the company encourages replacement of feeder cells in hybridoma preparation with interleukin 6. Boehringer Mannheim's 1989 Catalogue contains

- McGarrity, G.J. *Adv. Cell Culture* **2**, 99-131 (1982).
- DeGuidice, R.A. & Gardella, R.S. *Tissue Culture Association Monograph* **5**, 104-115 (US Tissue Culture Association, Gaithersburg, Maryland, 1984).
- Chen, T.R. *Expl Cell Res.* **104**, 255-263 (1977).
- Russell, W.C., Newman, C. & Williamson, D.H. *Nature* **253**, 461-462 (1975).
- Barile, M.F. & Grabowski, M.W. *Meth. Mycoplasma*, **2**, 173-181 (1983).
- McGarrity, G.J. & Carson, D.A. *Expl Cell Res.* **139**, 199-205 (1982).
- Gabridge, M.E., Lundin, D.J. & Gladd, M.F. *In Vitro Cell Dev. Biol.* **22**, 491-498 (1986).
- Hay, R.J. *Analyt. Biochem.* **171**, 225-237 (1988).
- Jeansson, S. & Brorson, J.K. *Expl Cell Res.* **161**, 181-188 (1985).
- Van Diggelen, O.P., Shin, S. & Phillips, D.M. *Cancer Res.* **37**, 2680-2687 (1977).
- Schimmelpfeng, L., Langenberg, U. & Peters, H.J. *Nature* **285**, 661-662 (1980).
- Marcus, M., Lavi, U., Nattenberg, A., Rottem, S., & Markowitz, O. *Nature* **285**, 659-661 (1980).
- Hay, R.J. *Prog. Cancer Res. Ther.* **29**, 3-21 (1984).
- Borup-Christensen, P., Erb, K. & Jensen, J.C. *J. Immunol. Meth.* **110**, 237-240 (1988).

more information on other cytokines and the company's complete range of cell and tissue culture reagents.

Researchers can turn tap water into reagent-grade water with the new RGW-5 Photronic water system, says Bellco (*Reader Service No. 107*). The system produces 18-megohm, bacteria-free water, at a rate of up to two litres per minute, by using five purification steps, including carbon adsorption, triple deionization and submicron filtration. The \$1,685 (US) unit has no moving parts, requires a 30-second rinse time, is available in left- and right-handed models and comes with an optional digital purity meter or purity lights.

The Protector Work Station safely ventilates toxic vapors while providing a convenient work area for staining and cover-slipping, says Labconco (*Reader Service No. 108*). The histology work station comes in models with or without several

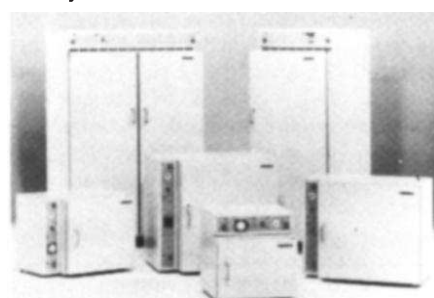


The Labconco histology work station. exhaust options, including a built-in blower or a roof-mounted blower. For laboratories without access to an outside duct, models with built-in charcoal filters are available that adsorb xylene, alcohol and formaldehyde. The basic station is four-feet wide and 24-feet high; a work surface and stand with molded epoxy sink, gooseneck water fixture, faucets and spray nozzle are optional.

LEEC Ltd. is announcing a new technology in cooled incubators (*Reader Service No. 109*). The LT series of incubators uses an entirely new temperature control system that overcomes the need to regularly defrost the units, says LEEC. The systems are capable of controlling chamber temperature at better than $\pm 0.1^\circ\text{C}$ over its 0 - 50°C range by blending airflows between hot and cold ducts. The units feature LED digital temperature displays together with over- and under-temperature safety thermostats. The outer cabinets of the incubators are manufactured from zinc-coated steel, and the

chambers are constructed from plastic with stainless steel ducting.

A new range of incubators that offers a variety of chamber sizes and a choice of



The Series 6000 natural convection incubators from Heraeus eliminate fan turbulence problems.

modular facilities has been introduced by Heraeus (*Reader Service No. 110*). The Series 6000 natural convection incubators are free from fan-produced air turbulence. Supplied for temperatures up to 50 or 70°C , the units have electronic control and spatial and time-period temperature stabilities. Application-specific modules include adjustable fresh air supply, automated programming, and RS232/V24, RS485 and 4-20 mA interfacing for external recorders and controllers. To suit individual needs, modules can be integrated at the time of purchase. The costs of incubators in the Series 6000 range from £530 (UK) to £1,785 (UK).

Culture conveniences

Flow Laboratories says its new Duacult roller bottle offers users $1,750\text{ cm}^2$ of surface area (*Reader Service No. 111*). The Duacult's external dimensions are the same as an 850-cm^2 bottle, but Flow says they have twice the internal surface area. The bottles are made from plasticizer-free polyester, for enhanced stability under exposure to radiation. The insides of the bottles are surface-treated to provide cell attachment for a wide range of cell lines, removing the need to pre-coat. A polyethylene liner is provided in the cap to create a secure seal, even when reduced



The $1,750\text{ cm}^2$ of surface area gives cell cultures more room to grow in.

temperatures cause a vacuum in the bottle. According to the company, the Duacult (£120 (UK) for a box of 20) also tolerates freezing without becoming brittle like polystyrene.

Nalge's Nalgene Media-Plus serum filter units now come with 90-mm nylon membranes (*Reader Service No. 112*). According to the company, the units are the first of their kind to be offered on the

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Reader Service No.37



The Media-Plus serum filter units from Nalge now come with 90-mm nylon membranes.

market. The nylon membranes have a 0.2- μm pore size, and are designed for use in tissue culture applications with sensitive cell lines, or in situations in which a wetting agent may cause problems. The nylon Media-Plus filter units are radiation-sterilized and cost \$192 (US) for a case of 12.

Three types of CelPrep gel entrapment media are now available from FMC Bio-

Correction

THE product review "Avoiding false positives with PCR" (*Nature* 339, 237; 18 May, 1989) was unfortunately published without the authors' final corrections and contains errors of emphasis and fact. First, the authors did not fully agree with the editor's summary that "tidiness and adherence to a strict set of procedures could prevent disaster", but preferred the idea that common sense and an awareness of these procedures could help prevent mistakes. Second, although the number of potentially contaminating molecules produced by PCR is large, the authors had intended to point out that there is an analogous situation in routine microbiology, where such standard operating procedures allow one to cope. Third, the authors intended to emphasize that controlling contamination entails thinking about the *relative* numbers of target molecules in samples handled together and the consequences of inadvertent physical transfer between them. Finally, it was to have been suggested that one should use only the number of PCR cycles necessary to achieve desired sensitivity and avoid the amplification in negative controls of inconsequential levels of contamination.

Other persisting errors include the number of molecules left in 0.1 ml of a dilution into an Olympic-sized swimming pool (40 not 400); the number of copies of a single-copy gene in one μg of human DNA (3×10^6); and reference and typographical errors. Also, it was not intended to suggest that UV lights be used in a biosafety hood *while* manipulating samples, but only between uses of the hood. Reprints of the article in corrected form are available from the authors. □

products (*Reader Service No. 113*). FMC Bioproducts says its CelPrep Alginate, CelPrep Agarose and CelPrep Carageenan provide different cellular environments and enhance tissue culture growth. Gel entrapment offers a three-dimensional cell growth matrix which can be used with a variety of cell types. The company tests each medium to ensure low endotoxin levels and freedom from mycoplasma contamination. Each gel medium is available in 125-ml quantities for \$30 (US).

To create a two-compartment system for transport studies, co-culture, chemotaxis and invasion assays, Costar recommends its Transwell **cell culture chamber insert** (*Reader Service No. 114*). The inserts provide a microporous basis for cell attachment, and are available with either polycarbonate or collagen-treated membranes. Polycarbonate membranes are available in pore sizes of 0.1 μm , 0.4 μm , 3.0 μm , 5.0 μm and 8.0 μm , with diameters to fit 24-well and 6-well plates. The collagen-treated, transparent membranes offer increased visualization of cell outlines during phase contrast microscopy, says Costar. The collagen-treated membranes cost \$131 (US) for 24-well plates and for \$143 for 6-well plates.

Sliced and diced

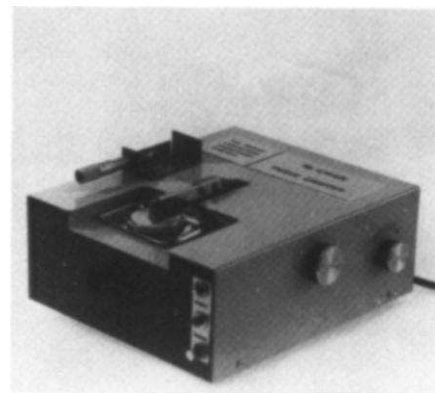
Biospec is introducing a **portable rotor-stator homogenizer** that can disperse, homogenize and emulsify samples of volumes between 0.5 ml and 50 ml (*Reader Service No. 115*). Suspended material is drawn into the homogenizer



Biospec's portable rotor-stator tissue homogenizer recharges in three hours.

probe by a screw-shaped rotor turning at high speeds. The suspension is then repeatedly recycled through six close-tolerance slits at a rate of 20,000 times per minute. Biospec says the rotor-stator homogenizer disintegrates and disperses most materials in one minute or less. The cordless, two-speed, hand-held instrument is rechargeable in three hours. Its probe is four inches in length and 0.25 inches in diameter.

A slicer for finely cutting biopsy tissues with less cellular damage than homogenizers is being introduced by Balzers (*Reader Service No. 116*). The Balzers **tissue slicer** is used for mechanically cut-



The tissue slicer from Balzers also makes sections, cubes and wedges.

ting tissues originating from biopsy or very small organs for subsequent embedding or ultramicrotomy. The instrument is capable of sectioning tissue as thin as 15-20 μm , says Balzers. The sectioning thickness of the slicer can be set in microns using its calibrated micrometer head, a property Balzers says is useful for antibody labeling and peroxidase studies. The \$2,860 (US) bench-top unit can also make sections, cubes and wedges of tissue.

Cambridge Instruments is now selling the Reichert-Jung Cryocut 1800 **cryostat**, which features a stainless steel microtome (*Reader Service No. 117*). The microtome is incorporated into an ergonomically designed freezer cabinet and has a spindle feed assembly and roller-bearing slideway that can cut specimens in 1- μm sections. The Cryocut 1800's refrigeration unit provides finger-touch temperature control, and its motor-driven course feed responds with button control. The availability of both automatic and manual 12-minute defrost cycles offer frost-free operation. □

These notes are compiled by Cary Prince from information provided by the manufacturers. To obtain additional information about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.